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THE APPLICATION OF
ABSORPTION SPECTRA
To the Study of
VITAMINS, HORMONES AND COENZYMES

BY

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PREFACE

Great progress has been made since the first edition of this book was written and it has been necessary to rewrite and enlarge the whole work. I have made free use of diagrams from the literature, often without the consent of the authors. Knowing that permission would have been granted at once in time of peace, I wish to make a general acknowledgment here.

My laboratory is the creation of E. C. C. Baly, and its biochemical activity goes back to 1927 when I. M. Heilbron asked me to investigate the cholesterol absorption. Since then I have been indebted to many research students and outside collaborators, to all of whom I am grateful for happy associations. Throughout this period my assistant, R. H. Creed, has rendered invaluable service. To the Medical Research Council I am indebted for substantial help and encouragement, and I owe much to the friendly assistance of my colleagues on the Vitamin A and Vitamin E sub-committees. In this field, the relations between academic, official and industrial bodies are unusually close and fruitful, and I wish to record my thanks for much help.

It is reasonable to ask why "Absorption Spectra" and "Vitamins and Hormones" should be bracketed in a book. The answer is that in the last twenty years the subject of Absorption Spectra has ceased to be a minor and auxiliary speciality within Chemistry and has become a very versatile tool, almost indispensable in many fields of research. The subject thus affords a good vantage point. More and more workers having other main interests, find it necessary to make use of absorption spectra, and they need a certain minimum acquaintance with methods of experimentation and interpretation. Similarly, the spectroscopists (of whom there are several varieties) need to see their subject against a wider background. This book is a contribution to the work of liaison. It reviews many brilliant papers, distinguished by patient work, great skill and insight, but it is much more a record of small advances, the cumulative effect of which is prodigious in its implications. The great days of line spectra are over; with the developments of quantum theory in the first third of this century, sub-atomics has become neat and tidy. Similarly quantum mechanics is providing an adequate theory of the absorption spectra of simple molecules. A great clarification of the theory of larger molecules has begun to take shape and in the next few years the study of absorption spectra will play its part in a process destined to affect the outlook of all chemists.

I am indebted to Mr. T. L. Tippell and Mr. T. W. Goodwin for valuable help in proof-reading and indexing.

R. A. MORTON

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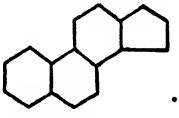
CHAPTER II

ABSORPTION SPECTRA AND STEROIDS

Steroids enter into fields of physiological and structural chemistry too vast to be properly reviewed in this work, and to give an adequate summary of recent research into the bile acids, sterols, hormones, cardiac aglucones and saponins would in any case be undesirable when it it desired to select topics for their relevance to spectroscopy.

The scope of this chapter will therefore be restricted so as to facilitate the correlation of absorption spectra and constitution by those wishing to make incidental use of spectrophotometry. This method of treatment will lessen the ease with which the chapter can be read, but it is hoped that some gain in usefulness will result. The monograph by Lettré and Inhoffen (*Ahrens' Sammlung*, 1936, vol. 29) provides a fuller background than is given here, and the *Annual Reviews of Biochemistry* and the *Annual Reports* published by the Chemical Society will guide the reader to wider original literature.

The study of vitamins and hormones has shown that biocatalysts differing strikingly in function are often structurally akin. The recurrence of a common skeleton is illustrated by the range of physiologically important substances derived from the fully saturated cyclo-pentano-perhydro-phenanthrene



In this as in other fields Nature seems to use molecular prototypes with rigid economy and to display its virtuosity in the evolution and elaboration of specific catalysts effective at very low concentrations.

The nomenclature, numbering of rings A B C and D and the side chains are illustrated in Chart I.

The occurrence of methyl groups at positions 10 and 13 is a very constant factor. The dimethyl derivative is *androstane*, a fully saturated polycyclic hydrocarbon. Introduction at position 17 of an ethyl group gives pregnane, of C_8H_{17} cholestane, of C_9H_{19} ergostane. The plant sterols, sitosterol and stigmasterol, have $C_{10}H_{21}$ and $C_{10}H_{19}$ respectively at position 17.

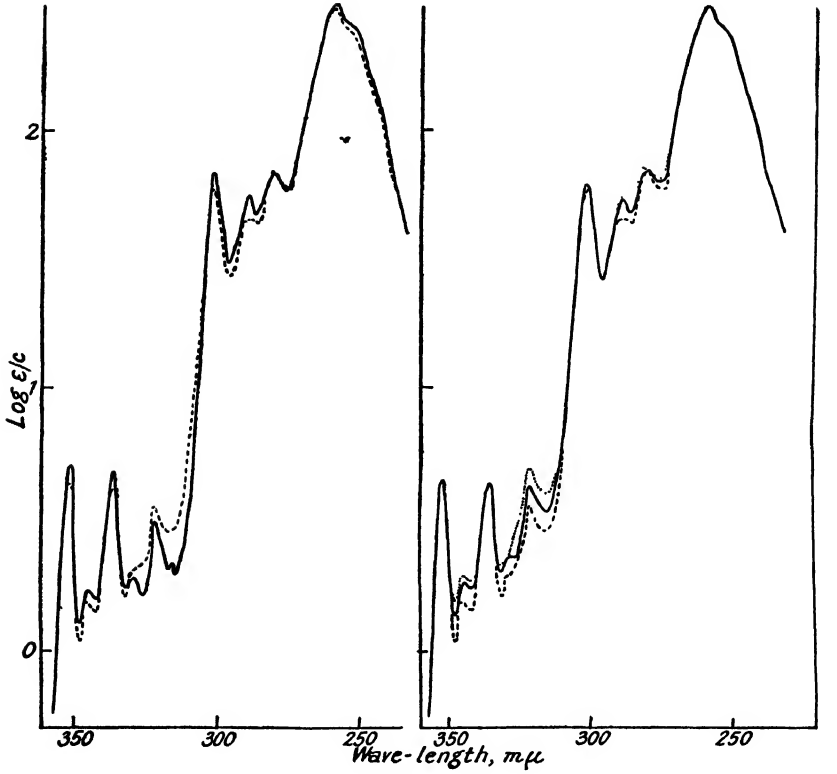
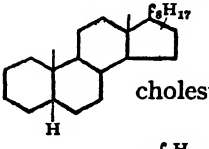


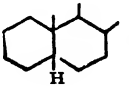
FIG. 4

——— *cyclo* Pentenophenanthrene — — — $C_{18}H_{16}$ from ergosterol.
——— $C_{18}H_{16}$ from cholesterol.
– – – 3-Methyl *cyclo* pentenophenanthrene.
After Harper, Kon and Ruzicka, *J.C.S.*, 1934, 124.

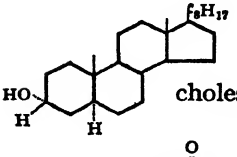
Numerous cases of isomerism occur thus :



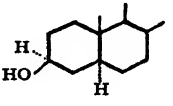
cholestane



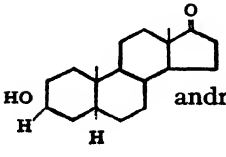
coprostane



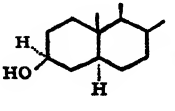
cholestanol



epi cholestanol



androsterone



trans androsterone

This kind of isomerism has little significance in determining absorption spectra when the chromophoric group is distant from the site of the steric difference but it is of great importance in the final elucidation of structure. The multiplicity of possible spatial configurations accounts for the numerous instances of isomerides, and the degradation of newly discovered materials to compounds of known configuration greatly assists in the determination of structure. The nomenclature is not difficult. The accepted abbreviations *en*, *ol*, *one*, etc., stand respectively for an ethenoid linkage, a hydroxyl and a carbonyl group.

Δ^4 or $\Delta^{4,5}$ implies a double bond connecting C_4 with C_5; $\Delta^{4,6}$ dien— implies two double bonds connecting C_4 with C_5 and C_6 with C_7 respectively. There are, of course, exceptions to the systematic nomenclature, *cf.* oestrone and oestriol (p. 42).

Before discussing the spectra of the steroids it is necessary to consider a number of simpler compounds. Fully saturated polycyclic hydrocarbons as well as paraffins are, for practical purposes, transparent over the spectral range 190mμ to 850mμ, but it is often difficult to obtain them so pure that all extraneous absorption has been eliminated. It is, however, safe to generalise that fully saturated substances show no characteristic absorption in the visible or ultra-violet regions amenable to study with a quartz spectrograph. A single double bond causes the appearance of an absorption maximum near 185mμ; $\epsilon_{max.}$ *ca* 10,000. Compounds of the type R'. CH=CH.R'' (R=alkyl) may show displacements of $\lambda_{max.}$ to as far as 215mμ if R is large, but there is relatively little change in $\epsilon_{max.}$ Introduction of –OH has little effect, so that the hydroxyl group is itself " transparent ". If the absorbing molecule contains two double bonds unconjugated, $\epsilon_{max.}$ will be approximately doubled but $\lambda_{max.}$ will be practically unaffected. When, however, the double bonds are conjugated the absorption is considerably displaced and $\epsilon_{max.}$ rises :

α-octadienoic acid $CH_3CH_2CH_2CH=CH-CH=CH\,.\,COOH$

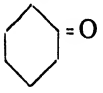
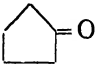
$\lambda_{max.}$ 260mμ, ϵ 26,500

β-octadienoic acid $CH_3CH_2CH=CH-CH=CH\,.\,CH_2\,COOH$

$\lambda_{max.}$ 228mμ, ϵ 24,400

The carboxyl group isolated from the conjugated double bonds by CH_2 has no effect on the position of $\lambda_{max.}$ and in saturated structures replacement of hydrogen by hydroxyl has no effect, *e.g.* both cyclohexane and cyclohexanol are transparent.

The carbonyl group constitutes a weak chromophore

	$\lambda_{max.}$	$\epsilon_{max.}$
$CH_3.CO.CH_3$	279mμ	14·4
$C_2H_5.CO.C_2H_5$	280	17·7
	289	14·8
	295	16·4
$(CH_3)_3.C.CO.C(CH_3)_3$	296	19·5

and fairly concentrated solutions (order M/10) are needed to record the absorption band; α- and β-diketones, R.CO.CO.R R.CO.CH_2.CO.R, likewise show feeble absorption unless there is enolisation.

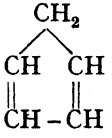
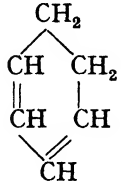
In αβ-unsaturated ketones, the low intensity carbonyl absorption is displaced and the ethylenic absorption resembles that of

R.CH=CH-CH=CH.R, *e.g.* Mesityl oxide $(CH_3)_2.C=CH-CO.CH_3$

$\lambda_{max.}$mμ	$\epsilon_{max.}$	solvent
327	40	hexane
229·5	12,600	hexane
315	55	methyl alcohol
238	10,700	methyl alcohol

In phorone (p. 24) both bands are strongly displaced, but enolic acetylacetone $CH_3C(OH)=CHCOCH_3$ has only one band ($\lambda_{max.}$275 mμ, $\epsilon_{max.}$10,000). Benzoylacetone (ketone form $C_6H_5.CO.CH_2.CO.CH_3$) shows three maxima 247, 280 and 320 mμ, $\epsilon_{max.}$13,000, 1000 and 50 respectively, whilst the enolic form shows $\lambda_{max.}$247 and 310 mμ, $\epsilon_{max.}$5600 and 15,000 respectively (see Morton, Calloway and Hassan, *J.C.S.*, 1934, 898). It is clear that the spectroscopic criterion for αβ unsaturated ketones must not be applied incautiously.

TABLE I

	$\lambda_{max.}$	$\epsilon_{max.}$	solvent
$CH_2=CH_2$	*ca* 180mμ	*ca* 10,000	
$CH_2=CH-CH=CH_2$	217mμ	21,000	hexane
$CH_2=C.CH_3-CH=CH_2$	220	24,000	hexane
$CH_2=C.Me-C.Me=CH_2$	225	20,000	hexane
$CH_3-(CH=CH)_4-CH_3$	272	4,200	hexane
	284	8,400	
	296	12,000	
	320	12,000	
$CH_3-(CH=CH)_6-CH_3$	330	5,800	chloroform
	340	10,600	
	360	16,000	
	372	11,400	
	239	3,400	hexane
	260	4,550	ether
$CH_2=CH-CH_2OH$	<195		hexane
$CH_3-(CH=CH)_2-CH_2OH$	<232		alcohol
$CH_3-(CH=CH)_3-CH_2OH$	268	53,100	alcohol
$CH_3-(CH=CH)_4-CH_2OH$	310	44,700	alcohol
$CH_3-CH=CH-COOH$	208	12,200	hexane
$CH_3-(CH=CH)_2-COOH$	261	25,200	hexane
$CH_3-(CH=CH)_3-COOH$	302	36,500	hexane
$CH_3-(CH=CH)_4-COOH$	330	49,200	hexane

More extended tables are to be found in an article by Dimroth (*Angew. Chem.*, 1939, **52**, 545).

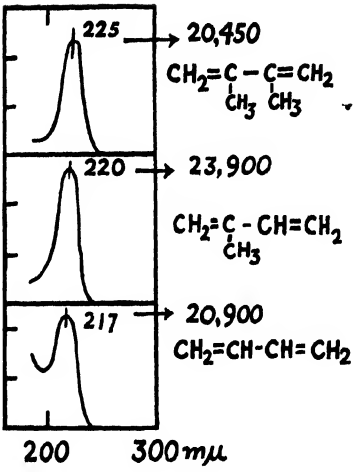


FIG. 5

Dimethylbutadiene
Isoprene
Butadiene

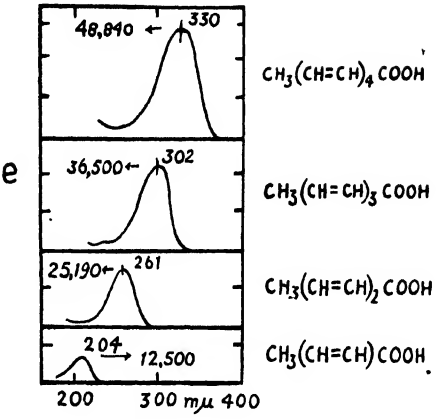


FIG. 6

Decatetraenoic acid
Octatrienoic acid
Sorbic acid
Crotonic acid

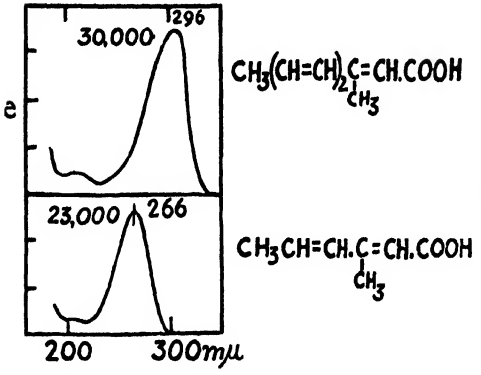


FIG. 7

Methyl octatrienoic acid
Methyl sorbic acid

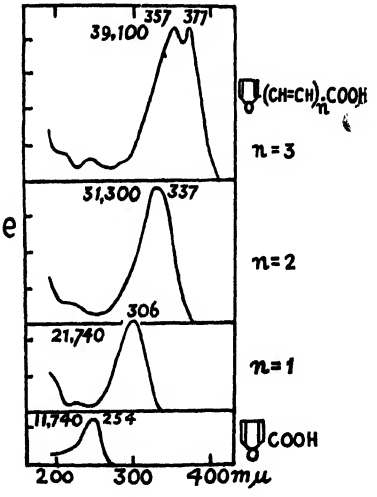
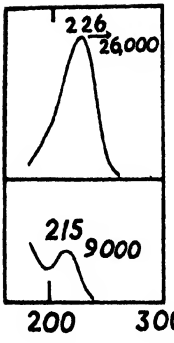
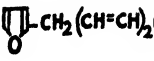


FIG. 8

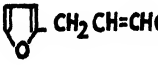
Furyl heptatrienoic acid
Furyl pentadienoic acid
Furyl acrylic acid
Furoic acid

Absorption spectra in hexane of poly-enes.
After A. Smakula (*Angew. Chem.*, 1934, **47**, 617)



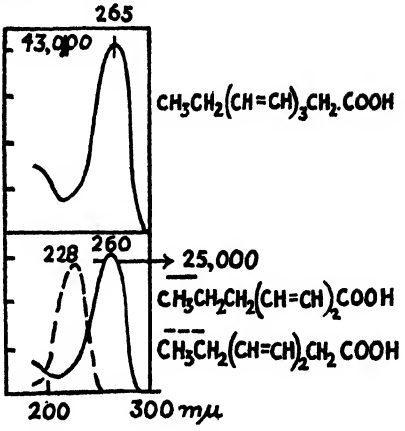


Furyl heptadienoic acid



Furyl $A\beta\gamma$-pentenoic acid

FIG. 9

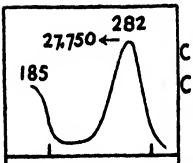


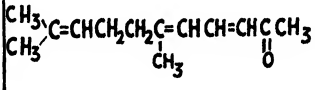
Decatrienoic acid

Octadienoic acids

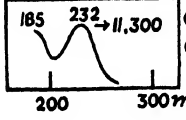
FIG. 11

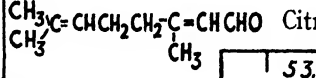
FIG. 10



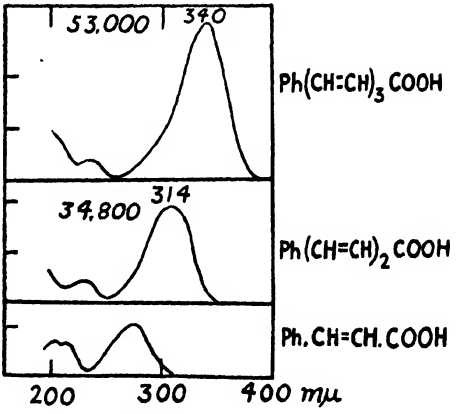


ψ Ionone





Citral



Phenyl heptatrienoic acid

Cinnamylacrylic acid

Cinnamic acid (alcoholic solutions)

FIG. 12

Absorption spectra of poly-enes (in hexane except for Fig. 12)
(After Smakula)

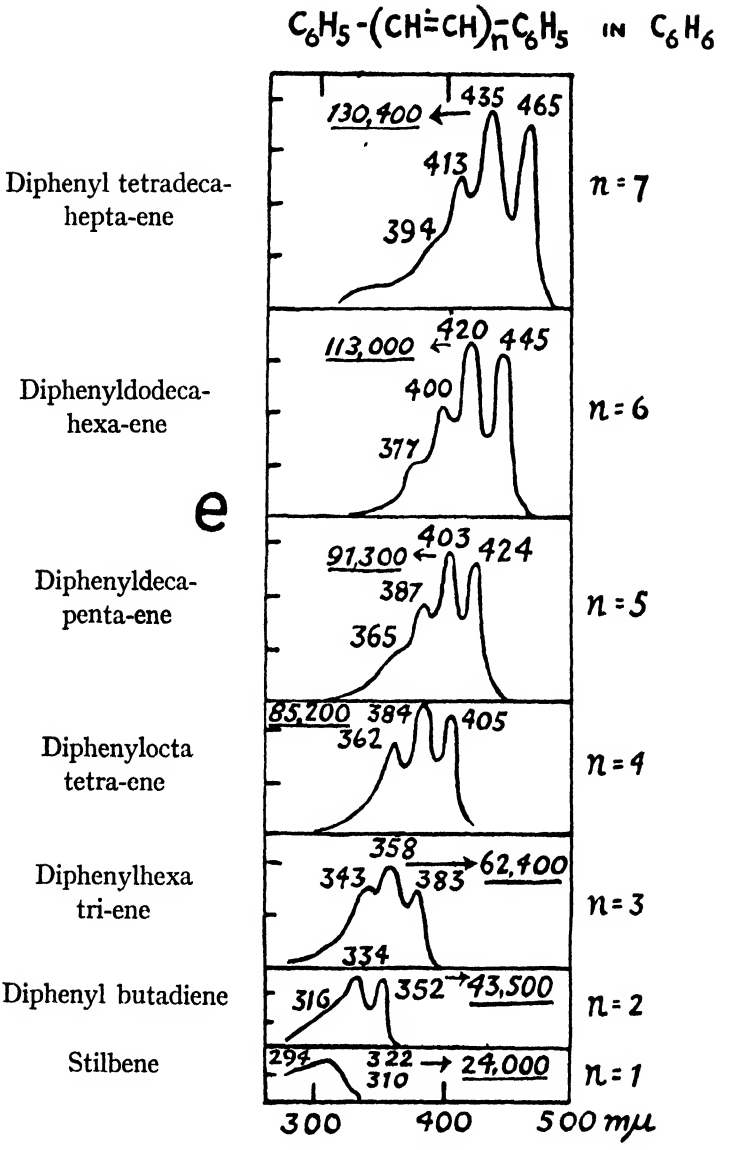


FIG. 13

Absorption spectra of diphenyl-polyenes.
(Adapted from Smakula).

cis-trans isomers

After Smakula and Wassermann.

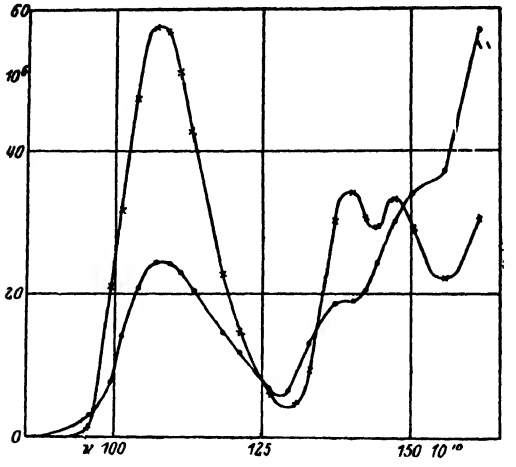


FIG. 14

Cinnamic acid in hexane

-•-•-•- *cis* form

-x-x-x- *trans* form

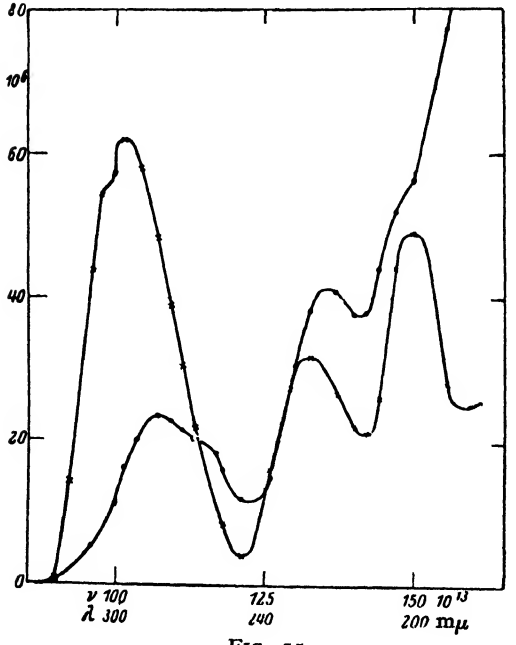


FIG. 15

Stilbene in hexane

—•—•—• *cis* form

-x-x-x- *trans* form

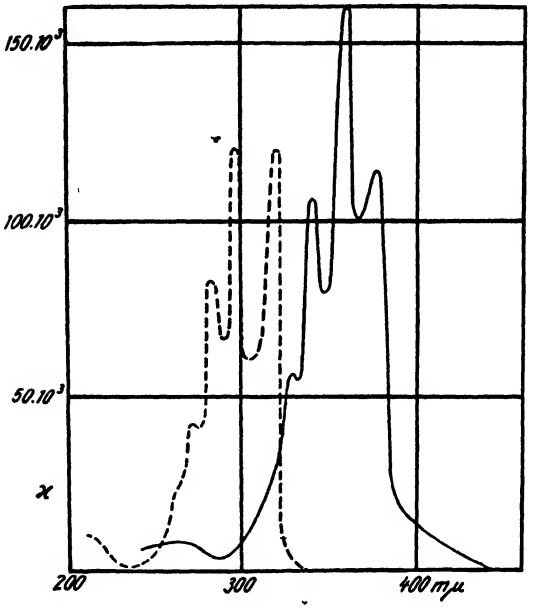


FIG. 16

$H_3C-(CH=CH)_6-CH_3$ ——— in Chloroform
$H_3C-(CH=CH)_4-CH_3$ ------ in Hexane

After Hausser

Replacement of R by OH or COOH may thus have quite marked effects on the poly-ene absorption.

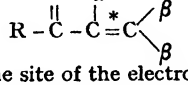
It is noteworthy that a break in conjugation

$$R-(CH=CH)_n.CH_2CH_2(CH=CH)_m.R$$
$$\quad a \qquad\qquad\qquad b$$

results in a summation of the effects due to *a* and *b*.

The low intensity band of α-β unsaturated ketones is not always easy to record because the substance may not be sufficiently soluble.

The ethylenic absorption

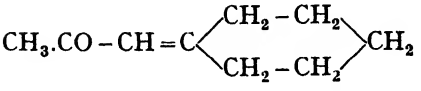
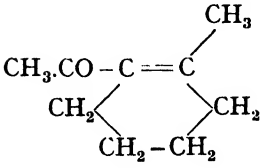
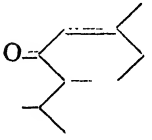
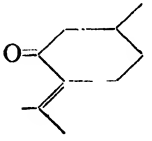


(* denoting the site of the electronic transition)
is displaced by induction to an extent depending on the degree of substitution.

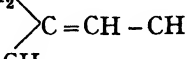
Substitution		$\lambda_{max.}$ mμ	number of substances tested
mono-	α or β	225 ±5	6
di-	αβ or ββ	239 ±5	36
tri-	αββ	254 ±5	9
ββ = H, H,		218	

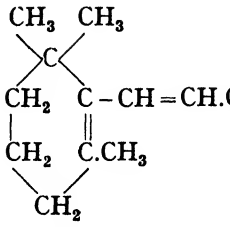
The above table (due to Woodward, *J. Amer. Chem. Soc.*, 1941, **63**, 1123; see also Evans and Gillam, *J. Chem. Soc.*, 1941, 815) is of obvious diagnostic value.

Substance (dissolved in alcohol) :

	$\lambda_{max.}$	$\epsilon_{max.}$
$CH_3.CO-CH=CH-CH_3$ - -	220mμ	10,400
	242	10,700
 - - -	234	31,300
 (cryptone) -	226·3 314	12·600 36·3
 (piperitone) -	235·3 319	15·850 54·6
 (pulegone) -	245 324	5·150 40

The chromophore $>C=\overset{|}{C}-\overset{|}{C}=\overset{|}{C}-\overset{|}{C}=O$ exhibits a further displacement of the ethylenic absorption :

	$\lambda_{max.}$	$\epsilon_{max.}$	solvent
$CH_3CO-CH=CH-CH=CH-CH_3$ -	271mμ	22,600	alcohol
$(CH_3)_2C=CH\,CH_2CH_2$  (ψ-ionone)	282	21,700	hexane



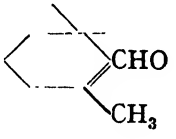
(β-ionone) - - 295

$(CH_3)_2C=CH-CO-CH=C(CH_3)_2$ (phorone) - 265·5 23,000; 355 120 alcohol

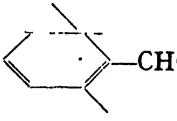
$CH_3CH=CH-CH=CH-CHO$ - - 270 26,500

$CH_3-(CH=CH)_3-CHO$ - - - 314 37,000 alcohol

- - - 228·5 18,600 alcohol



- - - 245 20,000



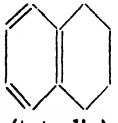
- - - 300 30,000

The carboxyl group is not itself a strong chromophore since formic, acetic and succinic acids show $\lambda_{max.}<210m\mu$ $\epsilon_{max.}$ 50–100

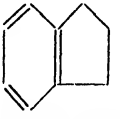
Aromatic substances like benzene and toluene show resolved absorption :

	$\lambda_{max.}m\mu$	$\epsilon_{max.}$		$\lambda_{max.}m\mu$	$\epsilon_{max.}$	
C_6H_6 - -	249	174	$C_6H_5CH_3$	255	183	(in hexane)
	255	210		263	234	
	261	144		270	229	

$C_6(CH_3)_6$ - 296 234 (in hexane)

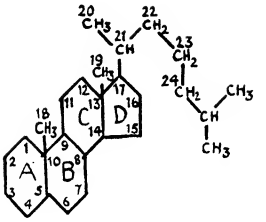


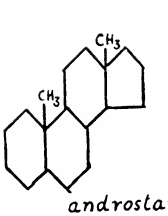
(tetralin) 267 740; 274 815



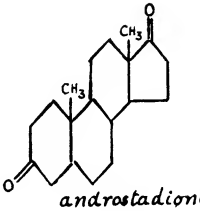
(hydrindene) 260 1000; 267 1450; 273·6 1780

CHART I

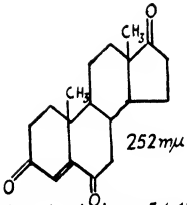




androstane

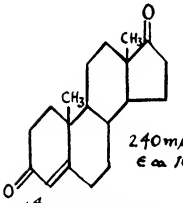


androstadione 3, 17



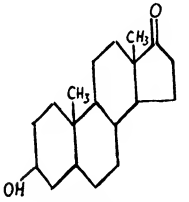
252mμ

Δ^4 androstentrione 3, 6, 17

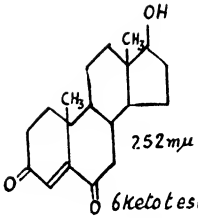


240mμ
ε ≈ 10,000

Δ^4 androstendione 3, 17
monosemicarbazone 275mμ

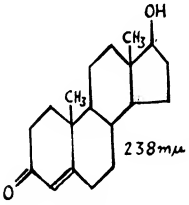


androsterone



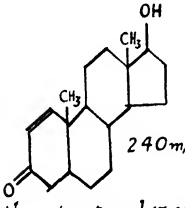
252mμ

6 keto testosterone



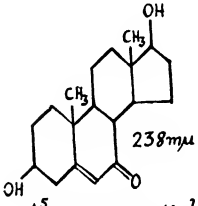
ε
238mμ 12,600

testosterone



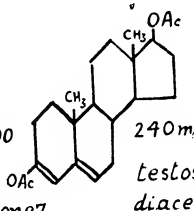
240mμ 5200

Δ^1 androstenol 17, one 3



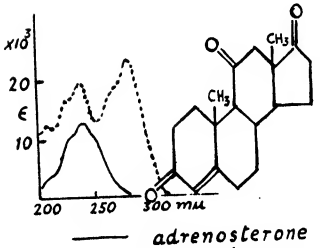
ε
238mμ 6,500

Δ^5 androstendiol 3, 17, one 7

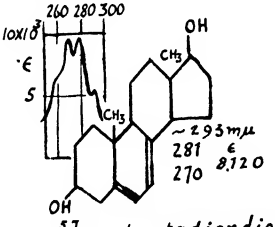


ε
240mμ 7,500

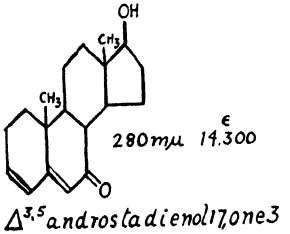
testosterone
diacetate



adrenosterone
disemicarbazone



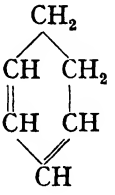
$\Delta^{5,7}$ androstadiendiol 3, 17.



ε
280mμ 14,300

$\Delta^{3,5}$ androstadienol 17, one 3

It is noteworthy that

	$\lambda_{max.}$	$\epsilon_{max.}$
$CH_3-(CH=CH)_3-CH_3$	260mμ	8000
and 	260mμ	4550

show maximum absorption at about the same wave-length as benzene but aromatisation reduces the intensity.

Introduction of hydroxyl or methoxyl causes some displacement and a considerable rise in ϵ_{max} but the acetoxy substituent makes little difference :

	$\lambda_{max.}$mμ	$\epsilon_{max.}$	solvent
phenol - - -	273	2400	methyl alcohol
anisole - - - -	271	2240	methyl alcohol
phenyl acetate - -	261	229	hexane

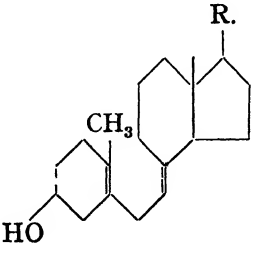
The foregoing principles provide the basis for interpreting the spectra of the steroids, but in the study of these large molecules several very interesting points have emerged which had not been foreseen from the data on simpler substances. Charts I, II, III and IV have been prepared to illustrate the data. I refers to androstane derivatives, *i.e.* those with no side chain at C_{17}, whilst IV refers to pregnane derivatives with a side chain containing two carbon atoms at position 17, and II summarises the information available for cholesterol derivatives with a side chain C_8H_{17}. Ergosterol derivatives have a side chain C_9H_{17} (one double bond C_{22-23}) at C_{17} (III); stigmasterol has the side chain:—$CH-CH=CH-CH-CH(CH_3)_2$, with CH_3 on the first CH and C_2H_5 on the second CH, *i.e.* $C_{10}H_{19}$, and the sitosterols have $C_{10}H_{21}$ at C_{17}.

All the fully saturated hydrocarbons, *e.g.* androstane, pregnane, cholestane, ergostane, etc., saturated hydroxy-bodies and unsaturated alcohols containing only one double bond, are highly transparent. Thus, a 10% solution of *pure* cholesterol in ether shows negligible absorption using a 4 cm. layer. Selective absorption on the long-wave side of 200mμ only appears in hydrocarbons when at least two conjugated double bonds

are present. If the two double bonds are in ring A as in $\Delta^{2,4}$cholestadiene (Bergman, *J. Org. Chem.*, 1936, **1**, 576), the absorption is of the same type as in cyclohexadiene. There are two clear maxima, viz. 267mμ and 275mμ, and $\epsilon_{max.}$ is 5500. The isomeric substances (with double bonds in ring B), $\Delta^{6,8}$ cholestadienol and $\Delta^{6,8}$ coprostadienol, show two similar maxima 270mμ and 280mμ, $\epsilon_{max.}$ 5350 and 4700 respectively (Windaus, *Ann.*, 1938, **534**, 23). The $\Delta^{5,7}$-derivatives show resolved absorption *ca* 280 mμ, with $\epsilon_{max.}$ of the order 10,000 (See Table II). When the two double bonds are not in the same ring the position of $\lambda_{max.}$ occurs at shorter wave-lengths.

	$\lambda_{max.}$mμ	$\epsilon_{max.}$
$\Delta^{3,5}$-cholestadiene	240	14,000 (Pallutz).
$\Delta^{4,6}$-cholestadiene	238	24,000 (Dane *et al.*, *Z. physiol. Chem.*, 1937, 245, 80).
$\Delta^{4,6}$-cholestadienol	238	8,300, *ibid.*
cholestadienol B	248	14,800 (Windaus, Linsert and Eckhardt, *Ann.*, 1938, **534**, 23, 536, 204).
7-methylene cholesterol	236	20,000 (Bann, Heilbron and Spring, *J.C.S.*, 1936, 1274).

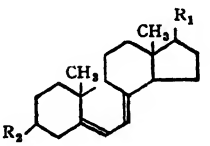
If the conjugation is broken as in dihydro-vitamins D_2 and D_3 (Windaus



and Roosen-Runge, *Z. physiol. Chem.*, 1939, **260**, 273) selective absorption in the region 200–250mμ disappears.

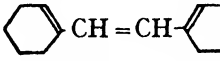
TABLE II

$\Delta^{5,7}$ derivatives.



R_1	R_2	$\lambda_{max.}$ mμ	$\epsilon_{max.}$	Provitamin D property (see p. 32)	
OH	OH	293 281 270	8,120 9,950	no	$\Delta^{5,7}$ androstadendiol 3, 17
C_8H_{17}	OH	293·5 281·5 271 260	11,900	yes	7-dehydrocholesterol (Windaus *et al.*, *Ann.*, 1935, **520**, 98)
$C_{10}H_{21}$	OH	260		yes	7-dehydrositosterol (Wunderlich, *Z. physiol. Chem.*, 1936, **241**, 176)
$C_{10}H_{19}$	OH	260		no*	7-dehydrostigmasterol (Linsert, *Z. physiol. Chem.*, 1936, **241**, 125)
C_9H_{17}	OH	260	12,400	yes	ergosterol
C_9H_{19}	OH	260		yes	22-dihydroergosterol (Windaus *et al.*, *Ann.*, 1933, **508**, 105)
$C_9H_{17}O$	OH	260		yes	ergosterol oxide (quoted, *Ber.*, 1939, **72**, 187)
C_9H_{17}	H	260	10,000		ergostatriene
C_9H_{17}	OH	270 280	10,000	yes	lumisterol (Windaus and Dimroth, *Ber.*, 1937, **70**, 576)
C_9H_{17}	OH	270 280	11,100		pyrocalciferol (*Ber.*, 1937, **70**, 576)
$C_5H_9O_2$	OH	293 281 270	6,300 15,800 12,600		*cis* 3-hydroxy $\Delta^{5,7}$ choladienic acid (Haslewood, *J.C.S.*, 1938, 226)

* This is unexpected.

Tachysterol (Fig. 15) is isomeric with calciferol, the spectrum shows little sign of resolution, and the ϵ value at $\lambda_{max.}$ is *unusually* high (*cf.*  $\lambda_{max.}$ 269mμ, $\epsilon_{max.}$ 22,900 ; Burkhardt and Hindley, *J.C.S.*, 1938, 987).

Ring closure (*e.g.* ring B) results in aromatisation and the absorption curves for *neo*ergosterol, *epi-neo*ergosterol, oestrone and oestriol revert to the types associated with benzene and tetralin and phenol respectively.

	λ_{max}	$\epsilon_{max.}$	
tetralin -	267mμ	740	(Morton and Gouveia, *J.C.S.*,
	274	815	1934, **911**, 916)

The low values for $\epsilon_{max.}$ are specially characteristic.

With four conjugated double bonds (Fig. 15) in ergostatri-en-one-enolacetate and ergostatetra-ene-one-enolacetate, the displacement of the resolved absorption of dehydroergosterol is carried a stage further in the direction of longer wave-lengths with persistence of high $\epsilon_{max.}$ values. In the case of tetradehydroergosterol a naphthalenic ring system is formed and there is a complete change in the type of absorption. The spectrum now shows a clear analogy with that of the (dimethyl) naphthalenes.

Simple ketones derived from *cyclo*pentano-perhydrophenanthrene do not seem to have been examined very frequently. They would be expected to show a typical ketonic absorption band of low intensity. Such a band is shown by androsterone (I and Fig. 23), λ_{max} *ca* 295mμ, $\epsilon_{max.}$ 4. A high extinction coefficient, if shown by a diketone, is a strong indication of enolisation provided that the molecule does not already contain ethylenic linkages of the types already discussed.

One of the simplest applications of absorption spectra to the steroids and one of the most useful (*cf.* Menschick, Page and Bossert, *Ann.*, 1932, **495**, 225) is the detection of $\alpha\beta$-unsaturated ketone structure (see also p. 18). An absorption curve similar to that of mesityl oxide may be expected and is shown typically by $\Delta^{4,5,17,20}$-pregnadien-3-one-21-al (IV, p. 46) where the ϵ values are doubled because two $-C=C-C=O-$ groups occur and are remote enough to be insulated from one another. It is not always possible to record the long-wave band. Sometimes it is difficult to obtain sufficient of the pure material, and at other times the solubility may not be sufficiently great to permit a solution of the necessary concentration to be prepared. Many workers have associated a band at *ca* 240mμ $\epsilon_{max.}$ 5,000–20,000 with $\alpha\beta$-unsaturated ketones without observing the band near 320mμ of low intensity. It is of course essential to support the spectroscopic evidence in other ways, such as the isolation of semicarbazones, etc. In order to avoid the uncritical use of the spectroscopic

criterion of $\alpha\beta$-unsaturated ketone structure it is important (*a*) to search for the 320mμ band and (*b*) to see that the absorption spectrum of the semicarbazone is displaced (*i.e.* 235–240mμ band replaced by a maximum *ca* 275 mμ) as in the spectra of the semicarbazones of ergosterone and *iso*-ergosterone.

By noting the value of $\epsilon_{max.}$ at 235–240mμ and comparing it with the values listed in Chart III, useful analogies may be drawn as to the spatial configuration exhibited by a new compound in this series, *e.g.* ϵ_{max} is 5200 at 240mμ for Δ^1-androstenolone-3 and 15,800 at 240mμ for cholestenone. The relevant configurations are discussed in the monograph by Lettré and Inhoffen already cited.

The chromophore $>C=C-C=C-CO$, which occurs in cholestadienone and *iso*-ergostatrienone, shows a band of very high intensity, ϵ_{max} 40,000 at 281mμ, but care should be taken not to regard such a high value as invariably associated with the grouping, thus for example androstadienolone shows $\epsilon_{max.}$ 14,300. When the chromophore is of the type shown in $\Delta^{1,4}$-cholestadienone, $>C=C-CO-C=C<$, the second double bond brings about no change in $\lambda_{max.}$, and ϵ_{max} is actually reduced :

	$\lambda_{max.}$ mμ	$\epsilon_{max.}$
Δ^4-cholestenone -	240	15,800
$\Delta^{1,4}$-cholestadienone -	235	11,300

A good example of the chromophoric inertness of a non-conjugated double bond is provided by comparing ergosterone, λ_{max} 235mμ, with *iso*-ergosterone, $\lambda_{max.}$ 275 and 330 mμ (III), the second (low intensity) band of the latter being due to the carbonyl group influenced by two conjugated double bonds. Another point arises with cholestan-2,3-dione. In alcohol, $\lambda_{max.}$ occurs at 272mμ, ϵ_{max} 5,000, whilst in ether, $\lambda_{max.}$ 268mμ shows ϵ_{max} 10,400. Such differences in intensity of absorption are always important and may, with caution, be used as criteria of keto-enol equilibria varying from solvent to solvent. It should be noted that the magnitude of the difference is here unexpectedly large. No attempt will be made in this work to carry the interpretation of steroid spectra very far in a fundamental sense. The field is rich in data, but unfortunately, the compounds have not always been available in a state of purity and the spectroscopic technique has not been uniformly good. The time therefore seems ripe only for the general kind of survey which has been made. Provided reasonable caution is shown, and steric effects allowed for in considering extinction coefficients (*cf.* Smakula, *Angew. Chem.*, 1934, **47**, 657, 777 ; Smakula and Wassermann, *Z. physikal. Chem.*, 1931, **155**A, 353 ; Lewis and Calvin, *Chem. Reviews*, 1939, **25**, 273), the tables shown in Charts II and III should assist in the identification of chromophores, and thus in elucidating structure.

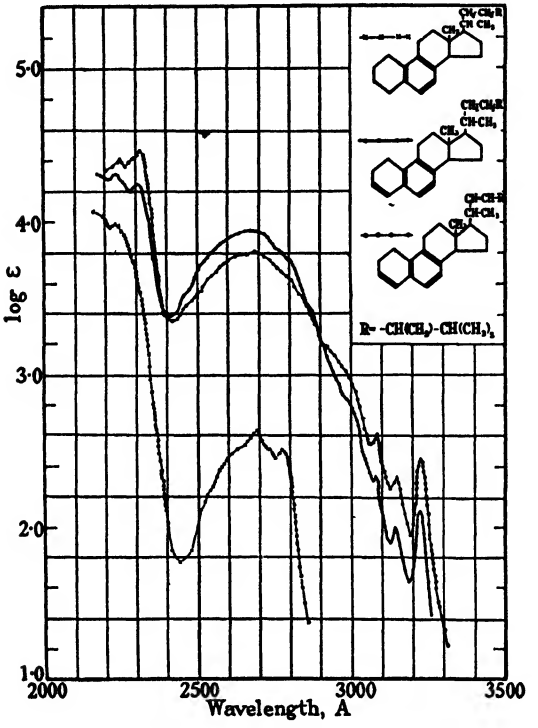


FIG. 17

× × × neoergostatriene.
• • • neoergostatetraene.
o o o neoergostapentaene
(After Mayneord and Roe)

Vitamins D and Provitamins D

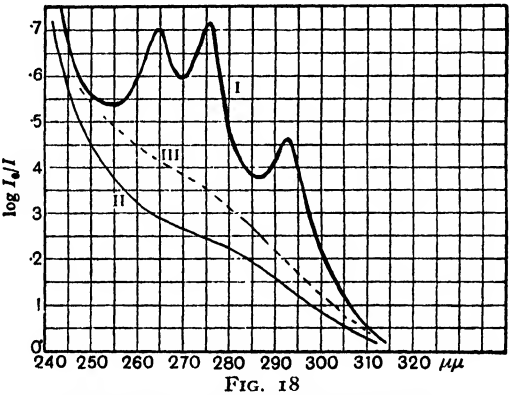
The faulty calcium- and phosphorus-metabolism which manifests itself in infants as rickets, and also occurs in young animals and birds, is largely due to wrong feeding (*cf.* Hess, *Rickets, Osteomalacia and Tetany*, Philadelphia, 1929). The importance of an accessory food factor (vitamin D), in this connexion has been known for some 20 years and the subject has occasioned a great deal of research. The literature is very fully surveyed by Heyroth in the new edition of Ellis and Wells' *Chemical Action of Ultra-Violet Rays* (Reinhold Publishing Co., New York, 1941).

There is probably no field of contemporary research with a more complicated background, and the pattern of knowledge now available depends upon the assembly of a multitude of facts, each the reward of effort and insight to which adequate tribute cannot be paid. The task of assessing the importance of each contribution is indeed a hopeless one and is moreover inconsistent with the purpose of this sketch.

Both the geographical distribution and the seasonal incidence of rickets are consistent with a preventive effect due in some way to sunlight (Schmorl, 1909) and more specifically to the rays of wave-length near 300mμ (Hess and Weinstock, *J. Amer. Med. Assoc.*, 1922, **20**, 14). About 1920 (*cf.* Huldschinsky, *Deut. Med. Wochschr.*, 1919, **45**, 712), it was widely recognised that exposure of the body to ultra-violet rays from sunlight or artificial sources tended to cure or prevent the disease. The most effective wave-lengths of artificial sources are near 280mμ (Knudson and Benford, *J. Biol. Chem.*, 1938, **124**, 287). E. Mellanby (*J. Physiol.*, 1918, **52**, XI) had already established the specific antirachitic activity of cod liver oil. No connexion was, however, then apparent between vitamin therapy and light therapy. It is now known that the skin contains a minute amount of a precursor substance (a provitamin D, almost certainly 7-dehydrocholesterol) which may undergo photochemical transformation, yielding a D vitamin which can be absorbed into the blood stream. Important work on irradiation of rats fed upon rachitogenic diets was carried out by Hume and Smith (*Biochem. J.*, 1923, **17**, 364; 1924, **18**, 1334), and a decisive step forward was taken when Steenbock, with Nelson and Black (*J. Biol. Chem.*, 1923, **56**, 355; 1924, **61**, 405; 1925, **62**, 275, 575; 1925, **64**, 263) showed that irradiation of the food could replace exposure of the animal itself to ultra-violet rays. They found that rachitogenic diets could become anti-rachitic after irradiation. Capacity to undergo such a photochemical "activation" was first found to reside in the fatty portion of food, then in the unsaponifiable fraction of the fat, and finally in the sterols "cholesterol" and "phytosterol".

The sterols so obtained appeared to be pure substances, they acquired quite considerable potency on irradiation, and there was obvious need for a thorough investigation of their absorption spectra and photochemistry. Hess and Weinstock (*J. Biol. Chem.*, 1924, **62**, 301; **62**, 297) found the absorption of irradiated cholesterol to be somewhat weaker than that of the unexposed sterol. Examining the latter, Schlutz and Morse (*Am. J. Dis. Child.*, 1925, **30**, 199) and Schlutz and Ziegler (*J. Biol. Chem.*, 1926, **69**, 415) observed two absorption maxima at 294 and 270mμ respectively. Heilbron, Kamm and Morton (*Chem. Ind.*, 1926, **45**, 932; *Biochem. J.*, 1927, **21**, 78) subjected large quantities of cholesterol to fractional crystallisation. They obtained evidence that cholesterol is an extremely transparent substance in the region 200–300mμ when pure, but that it normally contains an impurity which accumulates in the least soluble fractions; these show well-defined selective absorption with four characteristic maxima at 293·5, 281·5, 270 and 260mμ. The absorbing contaminant is the material which undergoes photochemical change. Different fractions showing absorption at widely different intensities were irradiated under

the same conditions and the products tested for antirachitic activity. A definite parallelism emerged between acquired potency and intensity of absorption in the unirradiated material. Hence it was concluded that cholesterol contained a trace of a photochemical precursor to vitamin D, since known as provitamin D. Pohl (*Nachr. Ges. Wiss., Göttingen*, 1926, **1**, 142; 1927, **2**, 185) arrived at similar results about the same time and Rosenheim and Webster (*Biochem. J.*, 1926, **20**, 537) found that at least 99% of the cholesterol was unaffected by irradiation. These workers (*Lancet*, 1925, **1**, 1025) had already recorded the fact that ergosterol (present in ergot of rye and in yeast) became antirachitic on irradiation. Later, it was found that the "activated" ergosterol possessed unexpectedly high potency, because the biological response was obtained



(After Heilbron Kamm and Morton)

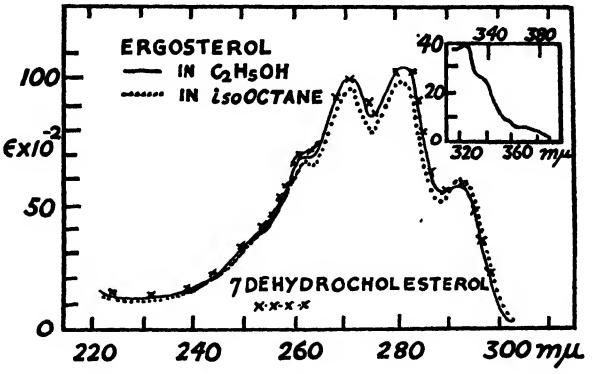
FIG. 18

I. Least soluble fraction from cholesterol.
II. Cholesterol after removal of active compound.
III. Irradiated fraction.

with very much smaller doses than had previously been tried. When the absorption spectrum of ergosterol was studied (Windaus and Hess, *Nachr. Ges. Wiss., Göttingen*, 1927, **2**, 173; Rosenheim and Webster. *Biochem. J.*, 1927, **21**, 111), it was found that the compound showed precisely the same absorption as the provitamin D of impure cholesterol, but that the intensity was $10^3 - 10^4$ times greater. It was natural to regard ergosterol as *the* progenitor of vitamin D, and although this view was subsequently shown to require modification, there was every reason to undertake intensive study of the photochemistry of ergosterol.

At this stage most workers expected the isolation of vitamin D to follow readily, but the irradiation process turned out to be very complicated. In the first place, the conditions of exposure need to be accurately specified if reproducible results are to be obtained. The variables include (*a*) the spectral energy-distribution of the light, (*b*) the optical properties of the solvent, (*c*) the time of exposure and distance from light source to

solution, and (*d*) the concentration. In the second place, the photochemical transformation of ergosterol involves several processes which may be consecutive or simultaneous, and it is never possible to obtain vitamin D as the sole product of irradiation. In fact, six or more substances may be present in the resin-like product obtained after removing the solvent. The sequence of changes has now been elucidated, largely as a result of the



After Hogness *et al.*

FIG. 19

work of the Göttingen school led by Windaus,* and of a team working at the National Institute for Medical Research.† Important contributions were also made by Reiter, Reerink and van Wijk,‡ and by Bills,§ as well as numerous other investigators. Various light sources were used, the mercury arc (filtered or unfiltered), and the magnesium spark (specially rich in radiations of wave-length 280–285mμ), being most favoured. At one stage, the irradiation product was subjected to distillation in a very high vacuum, and some workers made use of digitonin to separate the unchanged ergosterol from the photochemical resultants. Advantage was taken of the possibility of condensing irradiation products with maleic and citraconic anhydrides, but perhaps the most useful advance of all was the formation with 3, 5-dinitrobenzoyl chloride of recrystallisable esters from which pure alcohols could be regenerated. Absorption spectra studies and determinations of optical rotations played a useful part throughout.

Prolonged irradiation results in the disappearance of all the ergosterol but the vitamin D is also destroyed. If the process is interrupted at an earlier stage, the unchanged ergosterol may be removed as the digitonide, leaving an active but intractable resin. Oxygen must be excluded during exposure to ultra-violet rays and in the subsequent treatment. Highly

* Windaus, Linsert, Luttringhaus and Weidlich (*Ann.*, 1932, **492**, 226; Windaus, von Werder and Luttringhaus, *ibid.* 1932, **499**, 188; † Askew, Bourdillon, Bruce, Jenkins, Webster, Angus, Fischmann, Callow, Philpot (*Proc. Roy. Soc.*, 1929, **104B**, 561; 1930, **107B**, 76, 91; 1931, **108B**, 340). ‡ Reerink and van Wijk (*Biochem. J.*, 1929, **23**, 1294). § Bills, Honeywell and Cox, (*J. Biol. Chem.*, 1928, **80**, 557; 1931, **92**, 601).

potent preparations result when 40–75% of the ergosterol remains unchanged, but the active fraction is very difficult to crystallise even after esterification (acetate, palmitate, etc.) The active resin is in fact very much a mixture. Different workers, using various methods of irradiation, obtained products of similar potency which exhibited marked variations in spectral absorption and optical rotation. Windaus and his colleagues succeeded in eliminating inactive material by condensing it with maleic anhydride, and the residue yielded crystals of very high activity (vitamin D_1) from petrol ether.

EXAMPLE :—8 g ergosterol in 270 ml. abs. ether were irradiated in a quartz vessel for 9 hours (Hg. arc) ; 41% unchanged ergosterol was then removed by digitonin. 3·4 g of resin were left to stand in ether for 10 days with 3 g. citraconic anhydride. Separation into neutral and acidic fractions followed (45 and 55% respectively) and the neutral portion crystallised readily. On recrystallisation (acetone) 0·6 g. of vitamin D, *m.p.* 124–125° $[\alpha]_D + 140·5°$ (acetone) was obtained.

Meanwhile, crystalline preparations had been obtained in London by high vacuum distillation and Reerink and van Wijk had further purified their product. Treatment of the active distilled material with 3,5-dinitrobenzoyl chloride yielded a mixture of esters which could be separated into three fractions from which alcohols could be regenerated on hydrolysis :

		$\left[\alpha\right]^{20}_{546·1m\mu}$	3,5-dinitrobenzoates
	m.p.		*m.p.*
calciferol	114·5–117°	122·5° (alcohol)	147–149°
pyrocalciferol	93–95°	624° (alcohol)	167·5–169·5°
lumisterol	116·5–118·5°	220° (alcohol)	139·6–141·5°

Calciferol (now known as vitamin D_2) proved to be twice as active as vitamin D_1, whilst lumisterol and pyrocalciferol were inactive. Linsert had also isolated vitamin D_2, and after specimens had been exchanged between the London and Göttingen groups it was clear that vitamin D_1 contained lumisterol and calciferol in a 1 : 1 ratio. Experiments on rats showed that calciferol possessed the enormous antirachitic potency of 40×10^6 I.U. per gram as against 100–200 I.U. per gram for cod liver oil. When, however, equivalent quantities of calciferol and cod liver oil were supplied to chicks it was found that the vitamin D of fish liver oils was much more effective than an amount of calciferol equivalent in terms of rat units (Russell, Taylor and Wilcox, *J. Biol. Chem.*, 1934, **107**, 735 ; Dols, *Ann. Report Chem. Soc.*, 1935, 405). This and other biological evidence made it clear that more than one substance can elicit a "vitamin D" response.

Windaus, Lettré and Schenk (*Ann.*, 1935, **520**, 98) found that 7-de-

hydrocholesterol (prepared in the laboratory from cholesterol) exhibits the same absorption spectrum as ergosterol and, on irradiation, yields an antirachitically active product. 7-Dehydrocholesterol differs from ergosterol in having no C_{22-23} double bond and no methyl group at C_{24}. It is not therefore surprising to find that 22-dihydroergosterol is also a provitamin D. Windaus, Schenk and Werder (*Z. physiol. Chem.*, 1936, **241**, 100), using the method of chromatographic adsorption on aluminium oxide (Brockman), isolated from the irradiation product a vitamin D_3 analogous to calciferol, and later Windaus and Trautman (*ibid.*, 1937, **247**, 185) isolated vitamin D_4 from the irradiation of 22-dihydroergosterol.

Vitamin D_3 was isolated from tunny fish liver oil by Brockman (*ibid.*, 1936, **241**, 100) and proved to be specially active for chickens (Dols, *Z. Vitaminforsch.*, 1936, **5**, 161). Brockman (*Z. physiol. Chem.*, 1937, **245**, 96) also found that the antirachitic agent in halibut liver oil was vitamin D_3.

The provitamin present in crude cholesterol was isolated by chromatographic separation of acetates and proved to be 7-dehydrocholesterol (Boer, Reerink, van Wijk and Niekerk, *Proc. K. Akad. Wetensch., Amsterdam*, 1936, **39**, 662). Windaus and Bock (*Z. physiol. Chem.*, 1937, **245**, 168) found the sterol of pigskin to contain nearly 6% of provitamin D, again in the form of 7-dehydrocholesterol. The sterols from invertebrates (Bock and Wetter, *ibid.*, 1938, **256**, 33) generally contain ergosterol. It is probable that ergosterol is usually the provitamin occurring in vegetable products, at any rate the 7-dehydro-sito- and -stigmasterols are not found.

The sequence of changes which take place on irradiation of a provitamin is now clear: (*cf.* Windaus, Luttringhaus and Busse, *Nachr. Wiss. Göttingen*, 1932, 150; Setz, *Z. physiol. Chem.*, 1933, **215**, 183; Windaus, Deppe and Wunderlich, *Ann.*, 1937, **533**, 118; Windaus and Guntzel, *Ann.*, 1939, **538**, 120).

ergosterol (*provitamin D_2*)	7-dehydrocholesterol (*provitamin D_3*)	22-dihydroergosterol (*provitamin D_4*)
↓	↓	↓
lumisterol	lumisterol$_3$	lumisterol$_4$
↓	↓	↓
tachysterol	tachysterol$_3$	tachysterol$_4$
↓	↓	↓
calciferol	vitamin D_3	vitamin D_4
↓	↓	↓
Toxisterol	etc.	etc.
↓		
Suprasterols I and II and a substance with $\lambda_{max.}$ 248mμ		

The following table summarises the properties used in characterising the compounds:

TABLE III

Substance	m.p.	$\lambda_{max.}$ mμ	$E^{1\%}_{1\,cm.}$*	optical rotations
Calciferol 35·7–45 . 10^6 I.U./g.	116°	265	460–500 (460)	$[\alpha]^{20}_{5461}$ + 123·25°–125·75 (alcohol)
(Bacharach, Allchorne and Glynn, *Biochem. J.*, 1936, **30**, 2004).				
Vitamin D_3 40 . 10^6 I.U./g.	82–84°	265	500 (500)	$[\alpha]^{20}_{D}$ + 83·3° (acetone)
(Windaus *et al.*, *Ann.*, 1937, **533**, 118)				
Vitamin D_2 (from tunny liver oil)		265	(502)	
Vitamin D_4	107–8°	265	480 (470)	$[\alpha]^{18}_{D}$ +89·3°
(Windaus and Trautman, *Z. physiol. Chem.*, 1937, **247**, 185)				
7-dehydrocholesterol	142–3·5°	absorption spectrum similar to that of ergosterol in all respects		$[\alpha]^{20}_{D}$ −113·6°
(Windaus, Lettré and Schenk, *Ann.*, 1935, **520**, 98)				
Ergosterol	162–4°	281 (see p. 33)	∢320	$[\alpha]^{20}_{5461}$ −157° (chloroform) $[\alpha]^{20}_{D}$ −123·5°

* Figures in parentheses due to Brockman and Busse (*Z. physiol. Chem.*, 1938, **256**, 252).

Bock and Wetter (*Z. physiol. Chem.*, 1938, **256**, 33) have shown that the sterols of invertebrates are much richer in provitamin D than those of mammals.

	% Provitamin in sterol	
Worms		
Nereis virus (Rag worm) - - -	3·05	
Hirudinea officinalis (Leech) - -	3·5	
Arenicola marina (Lug worm) - -	3·68	2·7
Lumbricus terrestris (Earth worm) -	22·8	
Molluscs		
Mytilus edulis (Mussel) - - -	8·9	9·3
Helix pomatia (Vineyard snail) -	9·3	10·1
Arion empericon (Red slug) - -	19	25
Buccinum undatum (Whelk) - -	17·2	27·5
Arthropods		
Mehlwurm (Meal worm) - - -	15·4	
Daphnia (Freshwater flea) - -	0·75	

The provitamin D of invertebrates is nearly always ergosterol, but Buccinum undatum contains 7-dehydrocholesterol. There is no doubt about this, and it is interesting that nearly all the provitamin occurs in the alimentary tract. Windaus and Bock (*ibid.*, **256**, 47) have established that the sterol of wheat germ oil contains ergosterol as the sole vitamin D precursor.

To anyone accustomed to the low provitamin content of sterols from mammalian sources (*e.g.* 0·003% in halibut liver sterols and even less in bone marrow sterols) the high content of invertebrate sterols comes as something of a surprise. In examining the sterols prepared from the common earth worm by J. A. Lovern, the writer found about 25% of provitamin, and it seems that some so far unrecognisable rôle must be characteristic of the physiology of these substances. It is not very plausible to suggest that it is merely to provide for the vitamin D needs in calcium- and phosphorus-metabolism of *invertebrates*.

For a comprehensive survey see the new edition of Ellis and Wells (referred to earlier, p 30), Chap. 38, Rickets and Irradiation; Chap. 39, The photo-chemistry of the formation of Vitamin D; Chap 40, Commercial production of Vitamin D. Structural problems are, however, more fully reviewed in recent volumes of the Annual Reports of the Chemical Society.

Spectroscopic methods for determining vitamin D in natural products

Relatively little success has been achieved in the effort to find a satisfactory substitute for bio-assays as a means of determining the antirachitic potency of natural products. The difficulties are not necessarily insuperable and work is still in progress. Because of its interest and importance the problem will be considered in some detail.

The potency of pure calciferol is very high, *viz.* 40×10^6 I.U./g., whilst that of medicinal fish liver oils varies from *ca* 100 I.U./g. (cod liver oil) to 200,000 I.U./g. (tuna liver oil). This means that a successful analytical method must be as capable of determining 0·00025% as 0·5%. At all levels between these limits an accuracy of not less than $\pm$ 10% is desirable. Whatever procedure is tried, a preliminary concentration of the vitamin in the non-saponifiable fraction is essential and may vary from 10- to 100-fold. Even if this is achieved, the task does not lack difficulty, and it is not unexpected that only halting steps forward have been made. In the first place vitamin A occurs with vitamin D, accompanies it in the non-saponifiable fraction, and masks the vitamin D both in direct absorption and in colour tests. Provitamin D (presumably 7-dehydrocholesterol) also accumulates, and is very difficult either to eliminate or to estimate. Chromatographic methods are of the greatest value in attempting the isolation of vitamin D_3 from liver oil concentrates, but it is in the highest degree uncertain whether they can at present be applied quantitatively to this problem.

The most favourable conditions obtain in preparations relatively poor

in vitamin A and very rich in vitamin D. One such preparation yielded a non-saponifiable fraction with an inflexion near 325mμ and a strong band near 280mμ with definite indications of the three narrow bands at 293, 281 and 270mμ of 7-dehydrocholesterol. In spite of the fact that the original preparation contained 0·5%, and the non-saponifiable fraction some 10% of vitamin D, the 265mμ maximum of the vitamin was completely masked. The task of removing vitamin A and provitamin D quantitatively from vitamin D is a formidable precondition of success in the estimation of vitamin D by direct spectrophotometry. The method is at present inapplicable to natural products.

Several attempts have been made to develop a colour test using antimony trichloride in chloroform. Brockmann and Chen (*Z. physiol. Chem.*, 1936, **241**, 129) found that D vitamins give a pale yellow colour with the reagent, vitamins D_2 and D_3 behaving similarly to one another. Many workers who tried the method found it gave highly erratic results which were traced ultimately to the reagent, which must be prepared with dry, alcohol-free chloroform and used fairly quickly. Inadequate sensitivity made the whole procedure inapplicable to most fish oils.

Experience in the author's laboratory showed that cholesterol, ergosterol and calciferol all gave colour tests with the ordinary antimony trichloride reagent developing at different rates, and showing intensities which were insufficiently reproducible from day to day. The relative intensities were profoundly affected by the addition of acetic anhydride to the reagent, and under carefully chosen conditions the sensitivity of the reaction towards vitamin D could be greatly improved. With three constituents in artificial mixtures additive results could be obtained, but in natural products one at least, *e.g.* cholesterol, would first have to be determined by an independent method, such as precipitation with digitonin. Moreover, vitamin A would have to be eliminated.

A very recent paper by Nield, Russell and Zimmerli (*J. Biol. Chem.*, 1940, **136**, 73) makes an interesting advance. If the reagent is made with carefully purified chloroform and anhydrous antimony trichloride (15–30 g./100 c.c.; concentration not critical) and 1–4% (usually 3%) of pure acetyl chloride added, the resulting liquid gives a yellowish pink solution with as little as 2μg. (40 I.U.) of vitamin D. The coloured solution shows a very well-defined maximum at 500mμ and reaches its maximum intensity (at $E^{1\%}_{1cm.}$ 500mμ, 1800) within 30 seconds. This is important, as common congeners of vitamin D (cholesterol, etc.) also develop a similar band, but more slowly. The vitamin D colour persists for at least 5 minutes and there is satisfactory proportionality between concentration and colour-test intensity.

An advance in another direction has been made by Milas, Heggie and Reynolds (*J. Ind. Eng. Chem., Anal. Ed.*, 1941, **13**, 227). Some of their experiments might well be repeated using the $SbCl_3$-acetyl chloride reagent, and from many points of view they carried the empirical approach far beyond the limits of safety. Perhaps the important suggestion in their work is the preliminary treatment of concentrates with maleic anhydride in dioxan whereby vitamin A, carotenoids and polyenes generally, are attacked much more readily than vitamin D. The maleic anhydride adducts give no reaction with the antimony trichloride reagent. The writer has found that this suggestion, coupled with the use of the acetyl chloride reagent, represents real progress, but very much remains to be done before the position of bio-assays can be weakened by alternative methods. The quantitative elimination of cholesterol remains a major objective. One possibility is the formation of a definite insoluble lithium chloride addition compound (*m.p.* 140-2°) by mixing pyridine solutions of cholesterol and the salt (Zwikke, *Centralblatt.*, 1917, **110**, 77). Another is the formation of cholesterol digitonide ($C_{83}H_{138}O_{30}$, needles, *m.p.* 240°, decomp.) by addition of a 1% solution of digitonin in 90% alcohol to a hot alcoholic solution of cholesterol.

Digitonin is not easily accessible (1941) and is worth recovery. Boiling xylol extracts cholesterol only. The digitonide dissolved in pyridine yields digitonin on addition of ether, or when boiled with acetic anhydride yields acetylated sterol, extractable with ether (Schönheimer and Dam, 1933).

*Sex Hormones**

The female sex cycle is in some degree a periodic preparation of the uterus for pregnancy, and in menstruation the resulting products are swept away. Two phases, each controlled from the ovaries, can be recognised; the first is one of proliferation, bound up with the ripening of the follicles in the ovary, and the second is a secretory phase, coinciding with formation of the corpus luteum. Both processes depend upon hormones, the follicular hormone and the luteinising hormone, respectively. The direct evidence for this view is that women who have been castrated can menstruate, after consecutive treatment with the two hormones. The periodic changes in the ovaries are controlled by secretions from the hypophysis since in hypophysectomised animals the ovarian activity ceases. Implantation of anterior pituitary glands, however, restores ovarian function and in young animals greatly accelerates sexual maturity. Extracts prepared from the anterior lobe of the pituitary body have the same effect. Zondek and Aschheim postulated two gonad-stimulating hormones secreted by the hypophysis, one active in the ripening of the follicle and the other in

* Important substances such as adrenaline, thyroxin and insulin have not been dealt with in this edition because absorption spectra studies have played little part in recent work concerning them.

corpus luteum formation. Similar but probably not identical gonadotropic hormones appear in urine of pregnancy, and are perhaps elaborated in the placenta. In males with malfunctioning sex glands, there is increased production of the hypophyseal gonadotropic hormone and increased production of the urinary gonad-stimulating factor. This indicates a balanced mechanism for the normal activity of the sex glands and the anterior pituitary body. In fact, although in males there is no periodic sex cycle, the secretions of the hypophysis control secondary sexual characteristics as they do in women. The gonad-stimulating hormone of the female anterior pituitary body evokes the development of male sex glands. There is little evidence of sex specificity, but it is clear that the hormone acts only on sex glands since castrated animals do not respond to it. The secondary sex hormones belong to the steroids, whilst the gonadotropic hormones are not so far distinguishable chemically from proteins.

The activities of the anterior pituitary body are, however, not limited to the secretion of gonad-stimulating hormones. The hypophysis is essential for normal growth and metabolism, and for maintaining the functions of the thyroid, adrenal and mammary glands. Without it, the adrenal cortex and the pancreas function badly. A dozen or so hormone-regulating secretions have been postulated for the anterior lobe of the pituitary. It is central to the whole endocrine system, and is admitted to be the most important " source and target of active substances " in the body. Next in importance is perhaps the adrenal cortex.

The study of the physiology and chemistry of the products obtained from the pituitary body has resulted in an enormous literature and great advances have been made. Although by careful fractionation it is possible to separate the factors eliciting different biological responses, criteria of chemical homogeneity are lacking and it is doubtful whether the active agents have been obtained pure.

The growth hormone may be sufficiently concentrated for 3μg. to be measurably active. The product is labile to heat and to acid and alkali, and activity disappears on treatment with pepsin and trypsin. The concentrate gives protein reactions and an absorption spectrum $\lambda_{max.}$ 283mμ, $\lambda_{min.}$ 268mμ, typical of proteins. The active agent dialyses and is ultra-filterable at p*H*10 through collodion (porosity 30mμ). In female rats it induces excessive growth and incipient giantism (Freud, Dingemanse and Levie, *Acta Brev. Neerland. Physiol.*, 1939, **9**, 74).

The gonadotropic hormones have been prepared in very concentrated form, but such extracts as the writer has been able to examine show only the absorption spectrum of proteins. It seems clear that the richest preparations contain carbohydrate (6% in gonadotropic hormone from the hypophysis, 19% in hormone preparations from pregnancy urine), and

the presence of mannose and galactose is indicated by a positive orcinsulphuric acid reaction. The active substance may be a glycoprotein. The work of Bandow on the detection of carbohydrates by the spectra of solutions in concentrated sulphuric acid is of interest in testing glycoproteins (*Biochem. Z.*, 1937, **294**, 124; 1938, **296**, 105; 1938, **298**, 81).

The thyreotropic hormone cannot be at all easily separated from the gonadotropic agent, but there is no doubt that the two hormones are different and that both are related to the proteins. The corticotropic and adrenotropic agents, and also prolactin, the lactation-stimulating hormone, are less easily extracted from the anterior pituitary body than the gonadotropic and thyreotropic principles. For the latter, only mild treatment is needed, whereas for the former, some degree of degradation or autolysis of proteins of high molecular weight seems to be essential. There is some evidence that the thyreotropic hormone, although readily extracted from the fresh glands, is not a protein of high molecular weight and it may fall into the category of albumenoses. The smallest molecule is perhaps that of the adrenotropic agent.

So far as present knowledge goes, absorption spectra studies are of limited utility in the field of pituitary hormones. Like many enzymes, the hormones possess no recognisable prosthetic group; they are not conjugated proteins of the type of the yellow respiratory enzyme; they present no characteristic spectrum and there is no evidence even of *transparent* detachable groups. The absorption spectrum which does appear, is nearly always that of a typical animal protein and the only information which can be gained is an approximate estimate of the tyrosine and tryptophane content of the preparation. As the inert or irrelevant proteins show very similar spectra it is not surprising that there have been no authenticated instances of a parallelism between potency and intensity of absorption (*cf.* p. 181).

It is not impossible that the versatility of the hypophysis may be due less to a plurality of specific proteins than to a small number of proteins of high molecular weight capable of fission into fragments possessing highly specific properties. Whilst this is speculative (*cf.* Ludwig, *Angew. Chem.*, 1938, **51**, 487), it is not inconsistent with the modern view concerning the recurrence, at regular intervals throughout a polypeptide chain, of particular amino-acid residues.

The physiology of the secondary sex hormones rests fundamentally on the responses made by castrated experimental animals to chemical preparations from sex glands or urine. Chemical investigation directed towards the isolation, recognition and characterisation of active principles must of course be controlled and directed in the light of bio-assays. If

little attention is devoted here to the biological side it is only because, so much unequivocal information has been gained that it is reasonable, at this date, to take it for granted in this book.

In 1929–30, Doisy, Butenandt, Marrian and their colleagues, independently isolated from pregnancy urine chemical substances capable of inducing oestrus in castrated animals. Confusion was at first caused by the non-identity of some of the materials, but this was removed by the recognition that several related substances, now known as oestrone, oestriol, etc., are oestrogenic. The steroid nature of the hormones became clear from the following steps:

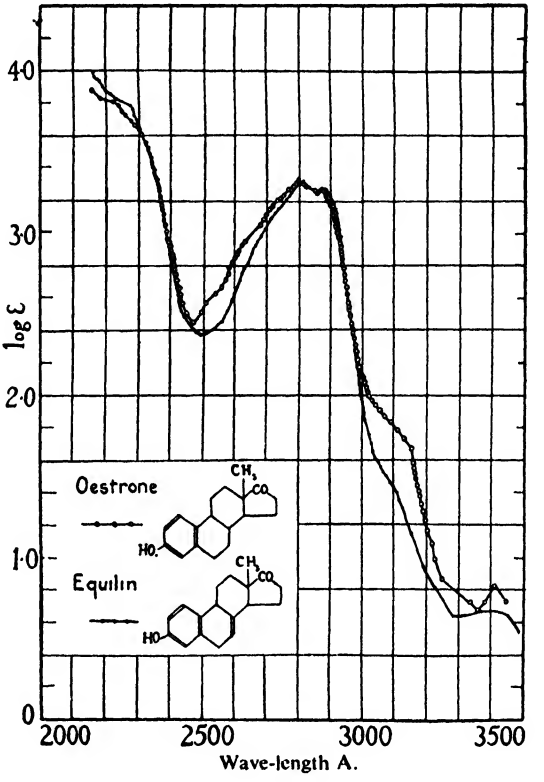
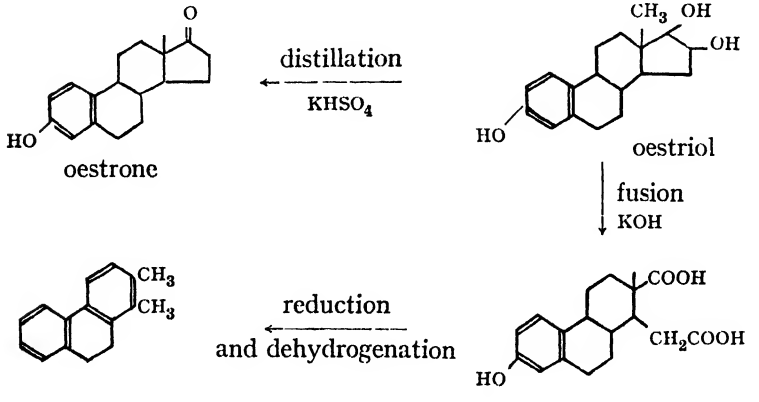


FIG. 20
After Mayneord and Roe



Girard, Sandulesco and Fridenson (*Comptes rend. Soc. Biol.*, 1933, **112**, 964; *Comptes rend. Acad. Sci.*, 1932, **194**, 909; 1933, **195**, 981) worked up enormous quantities of mares' urine and isolated three further oestrogenic substances of lower physiological activity: equilin, hippulin and equilenin. From 52 tons of pregnant mares' urine they isolated 1·5g. of the latter. Equilin and hippulin, $C_{18}H_{20}O_2$, and equilenin, $C_{18}H_{18}O_2$, were shown to be related to oestrone, $C_{18}H_{22}O_2$, and to differ by the possession of additional double bonds. They are all hydroxy (phenolic) bodies with

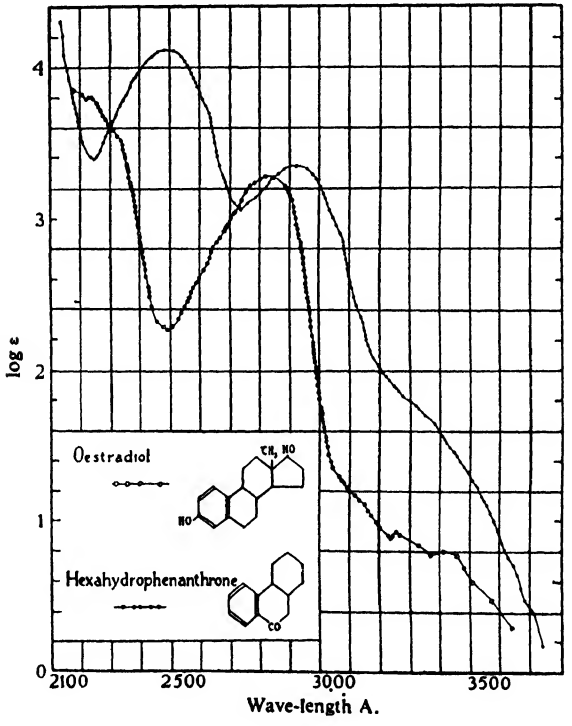


FIG. 21
After Mayneord and Roe

a carbonyl group, and both OH and CO are essential for physiological activity in this series. One exception must, however, be made; Schoeller Schwenk and Hildebrandt (*Naturwiss.*, 1933, **21**, 286) showed that dihydro-oestrone (*i.e.* oestradiol, $>C=O \xrightarrow{2H} >CHOH$) is about 6 times as potent as oestrone when tested on castrated mice.

Zondek showed that stallion testicles are extremely rich in oestrogenic material. Stallion urine is also a good source and it is estimated that quantities up to 50 mg. of hormone are produced per day. Oestrogenic

materials are also obtained from plant products, invertebrates, insects and protozoa. A large number of synthetic substances related only distantly to the cyclopentano-perhydrophenanthrene skeleton are also effective in some degree. There is therefore little specificity in oestrogens.

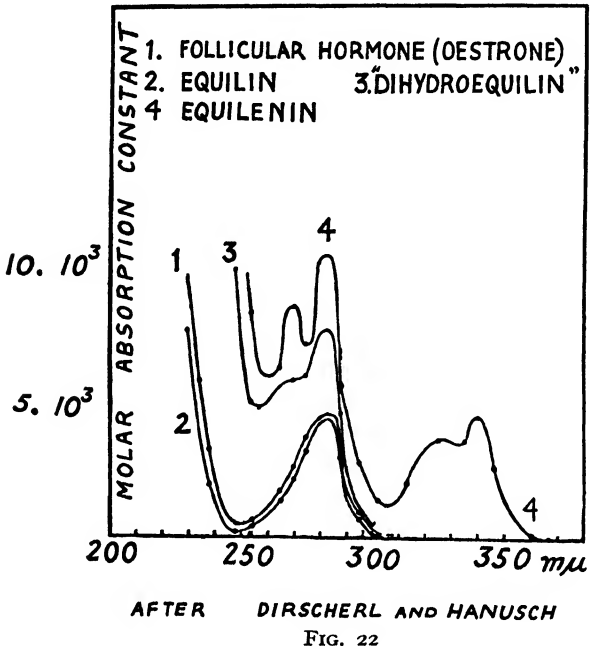
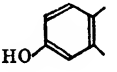


FIG. 22

The absorption spectra of oestrone, oestradiol and oestriol are dominated by the grouping :



Both in intensity and spectral location, the selective absorption is closely similar to that of phenolic bodies (*cf.* Fig. 42). The spectra of equilin and equilenin are quite different from one another, but the curves of oestrone and equilin are practically the same (Dirscherl and Hanusch, *Z. physiol. Chem.*, 1935, **233**, 13), namely, $\lambda_{max.}$ *ca* 284mμ, $\epsilon_{max.}$ *ca* 4,500 ; compare

	$\lambda_{max.}$ mμ	$\epsilon_{max.}$	
m-xylenol	284·5	1850	(Conrad-Billroth, *Z. physikal. Chem.* 1933, **20**, 222)
	281	1670	
	274		

The additional double bond of equilin cannot thus be conjugated to the benzene ring. This result agrees with the data on Δ^1-dihydro-

naphthalene, which absorbs much more strongly than the Δ^2-isomeride (Morton and Gouveia, *J.C.S.*, 1934, 919). The spectrum of equilenin shows the characteristic fine structure associated with naphthalenic compounds. Marrian and Cohen have isolated from human pregnancy urine a water-soluble complex containing 55% of oestriol. It is a glucuronide not differing from oestriol in the general character of its selective absorption.

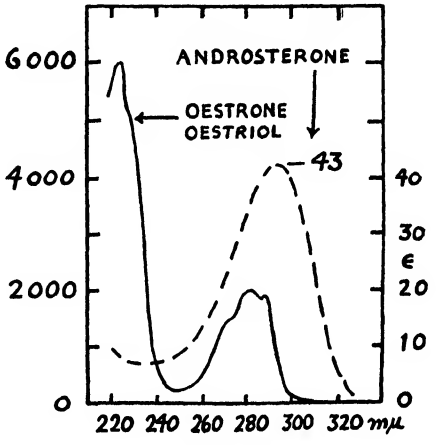
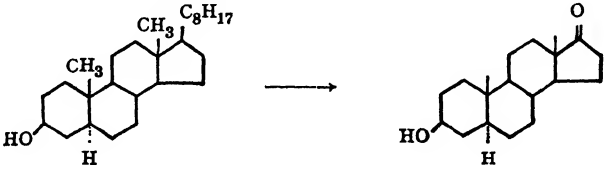


FIG. 23

The first isolation of a male hormone was achieved by Butenandt (15 mg. of pure material from 25,000 litres of male urine) in the shape of a ketone *androsterone* of *m.p.* 178°. One of the most convenient ways of testing for the presence of the hormone consists in measuring the increased comb growth in capons following administration of the preparation. 1 μg. exhibited a measurable response in this instance. Androsterone is a saturated hydroxy-ketone, $C_{19}H_{30}O_2$, and shows only the feeble selective absorption associated with the >C=O group. When the carbonyl group is reduced to >CHOH there is a marked increase in potency (*cf.* oestradiol).

Butenandt, Tscherning and Dannenbaum (*Z. physiol. Chem.*, 1934, **229**, 167, 185, 192) isolated three substances showing male hormone activity: androsterone, dehydroandrosterone-$C_{19}H_{28}O_2$ (1/3 as active as the saturated substance), and also an inactive chloroketone, $C_{19}H_{27}O.Cl$. Ruzicka, Goldberg and Brünnger (*Helv. Chim. Acta*, 1934, **17**, 1389, 1395) converted *epi*-dihydrocholesterol into androsterone:

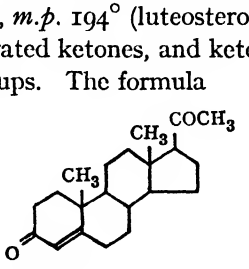


and found the product to be identical with the testicular hormone obtained from urine. Stereoisomerides of androsterone may be obtained by oxidation of coprosterol and *epi*-coprosterol acetates as well as dihydrocholesterol acetate.

isomeride derived from	active dose
(*a*) *epi*-dihydrocholesterol (*i.e.* androsterone) -	70μg. per day
(*b*) dihydrocholesterol - - - - - -	500μg.
(*c*) coprosterols *inactive* - - -	—

(*a*) and (*b*) differ only in the spatial position of the hydroxyl group.

Dehydroandrosterone does not belong to the *epi*- series but is active at 500μg. (comb growth), *i.e.* it shows about one-seventh the activity of androsterone, whilst judged by its effect on immature rats it is 1/3 as active. Androstandiol is 3 times as active as androsterone (comb growth). The active principle from testes was isolated by David, Dingemanse, Freud and Laqueur (*Z. physiol. Chem.*, 1935, **233**, 281). The substance now known as testosterone was soon recognised to be Δ^4-androstenol-17-one-3. It shows the typical spectrum of an $\alpha\beta$-unsaturated ketone. Testosterone is more effective than androsterone by the rat test and less active by the capon comb growth test. Androstendione-3,17 is also very active on rats (Ruzicka and Wettstein, *Helv. Chim. Acta*, 1935, **18**, 986 ; Wallis and Fernholz, *J. Amer. Chem. Soc.*, 1935, **57**, 1511, 2013 ; and later papers by Butenandt *et al.* on the specificity of male hormone action). The hormone secreted by the " yellow body " or corpus luteum of the ovary was obtained crystalline in 1930–33 (Fels and Slotta, *Klin Wochschr.*, 1930, **9**, 2004 ; Fevold, Hisaw and Leonard, *J. Amer. Chem. Soc.*, 1932, **54**, 254 ; Allen, *J. Biol. Chem.*, 1932, **98**, 591). The hormone may be separated from oestrone by utilising a phase separation between petrol ether and 33% alcohol, all the oestrone being extracted by the aqueous alcohol. Further work on the active material resulted in the separation of luteosterone-C, $C_{21}H_{30}O_2$, *m.p.* 128–129°, and luteosterone-D, $C_{21}H_{30}O_2$, *m.p.* 121° (Slotta, Ruschig and Fels, *Ber.*, 1934, **67**, 1270, 1624, 1947) and an inactive product $C_{21}H_{34}O_2$, *m.p.* 194° (luteosterone-A). The spectra were consistent with $\alpha\beta$-unsaturated ketones, and ketone reagents revealed the presence of two C=O groups. The formula



was suggested for the luteal hormone and a synthesis of pregnanol-20-one-3 from pregnandiol-3,20, followed by bromination and oxidation, gave a

monobromdiketone, which, on treatment with pyridine, gave the hormone of *m.p.* 121.° If solutions are seeded with the C form (*m.p.* 128·5). crystals of the same melting point are deposited. C and D therefore differ in crystalline form rather than chemical structure. The inactive material is pregnanol-3-one-20 (Butenandt and Mamoli). Pregnandiol, an inactive product from pregnancy urine, appears to be an excretory product derived from the hormones of the corpus luteum.

The name *progesterone* has now been accepted for the luteinising hormone :

(α-progesterone, *m.p.* 128·5° ; β-progesterone, *m.p.* 121°).

The following units have been adopted by the International Committee (League of Nations, Health Organisation) :

Oestrogenic hormones,

(*a*) oestrone, $C_{18}H_{22}O_2$; 1 I.U. = 0·1μg ; (*b*) oestradiol monobenzoate, *m.p.* 195° 0·1μg.

Male hormone,

androsterone, $C_{19}H_{30}O_2$, *m.p.* 183–183° ; $[\alpha]_D + 94°$; 1 I.U. = 0·1 mg. (capon comb growth).

Luteal hormone,

β-progesterone, *m.p.* 121° ; 1 I.U. = 1 mg. (uterine proliferation).

Adrenal Hormones

When the suprarenal glands are removed surgically, secretion of adrenaline decreases but there is a satisfactory response to injections, although the activity of the adrenal cortex is not so easily simulated. Absence of the active principle *cortin* causes, in the dog, an increase in the non-protein nitrogen (urea) of the blood, and in rats there is marked muscular fatigue resembling the distressing weakness shown in Addison's disease. (Swingle and Pfiffner, *Science*, 1930, **71**, 1321 ; *Medicine*, 1932, **11**, 371 ; Rogoff and Stewart, *J. A. Med. Assn.*, 1929, **92**, 1569 ; Hartmann and Brownell, *Science*, 1930, **72**, 76 ; Swingle and Pfiffner, *Am. J. Physiol.*, 1931, **98**, 144 ; *Proc. Soc. Exp. Biol. Med.*, 1931, **28**, 510 ; 1932, **29**, 144 ; Pfiffner and Vars, *J. Biol. Chem.*, 1934, **106**, 645 ; Wintersteiner *et al.*, *ibid.*, 1935, **111**, 585, 599 ; Kendall *et al.*, Mayo Clinic, 1934, **9**, 245). By working up large quantities of ox suprarenals, the latter groups of workers were able to isolate active crystalline materials, $C_{20}H_{30}O_5$, *m.p.* 210–213° and $(C_4H_6O)_6$, *m.p.* 208°.

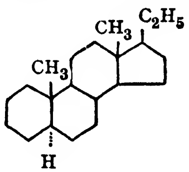
Reichstein and his colleagues have made very great contributions to the subject of the hormones of the adrenal cortex in a series of papers beginning in 1936 (*Helv. Chim. Acta*, 1936, **19**, 29) and not as yet completed. Whole ox suprarenals yield some 400mg. of raw extract per kg. When the extract is distributed between pentane and water (or aqueous HCl)

the active principles accompany the water-soluble constituents. From the aqueous phase they may be extracted by means of ether or ethyl acetate, the yield now being 30mg./kg. suprarenals. The use of $NaHSO_3$ and Girard's ketone reagents, permits a fractionation into ketones and non-ketones. These fractions are themselves heterogeneous, and the further purification procedures are too complicated to describe here (*cf.* Miescher, *Angew. Chem.*, 1938, **51**, 551; Reichstein, *Erg. Vitamin u. Hormonforsch.*, 1938, **1**, 334).

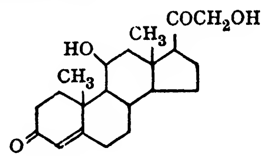
The Girard reagent T (*Helv. Chim. Acta*, 1936, **19**, 1095)

$$\underset{\displaystyle \mathrm{Cl}}{\underset{|}{(CH_3)_3N}} - CH_2 - CO.NH - NH_2$$

played a considerable part in Reichstein's work. Seven crystalline fractions were obtained, only one of which (F) showed selective absorption. It appeared to be an $\alpha\beta$-unsaturated ketone. Corticosterone, $C_{21}H_{30}O_4$, *m.p.* 180–182°, yielded allopregnane (*ibid.*, **21**, 161) in harmony with the structure more fully established later.

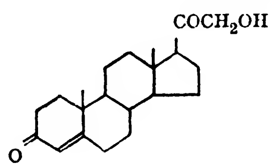


allopregnane



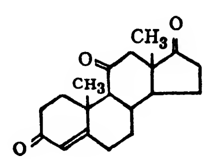
corticosterone

Six pregnane derivatives isolated from suprarenals are known to exhibit cortin activity, *viz.* corticosterone, dehydrocorticosterone, 17-oxy-



corticosterone, 17-oxydehydrocorticosterone, desoxy-corticosterone and substance S (*ibid.*, **21**, 1490). The tests used for measuring biological activity are difficult and do not agree too well among themselves, but it seems probable that desoxy-corticosterone is the most active material, and the simplest structure exhibiting the cortin effect. It is of course related to progesterone and might be designated 21-oxyprogesterone.

Another ketone from suprarenals, adrenosterone (*Helv. Chim. Acta*, 1936, **19**, 233) is of special interest in that it exhibits male hormone activity.



By the capon comb growth test it is 1/5 as active as androsterone. There are thus four substances bringing about this response : testosterone from testes, androsterone and *trans*-dehydroandrosterone from urine, and adrenosterone from suprarenals.

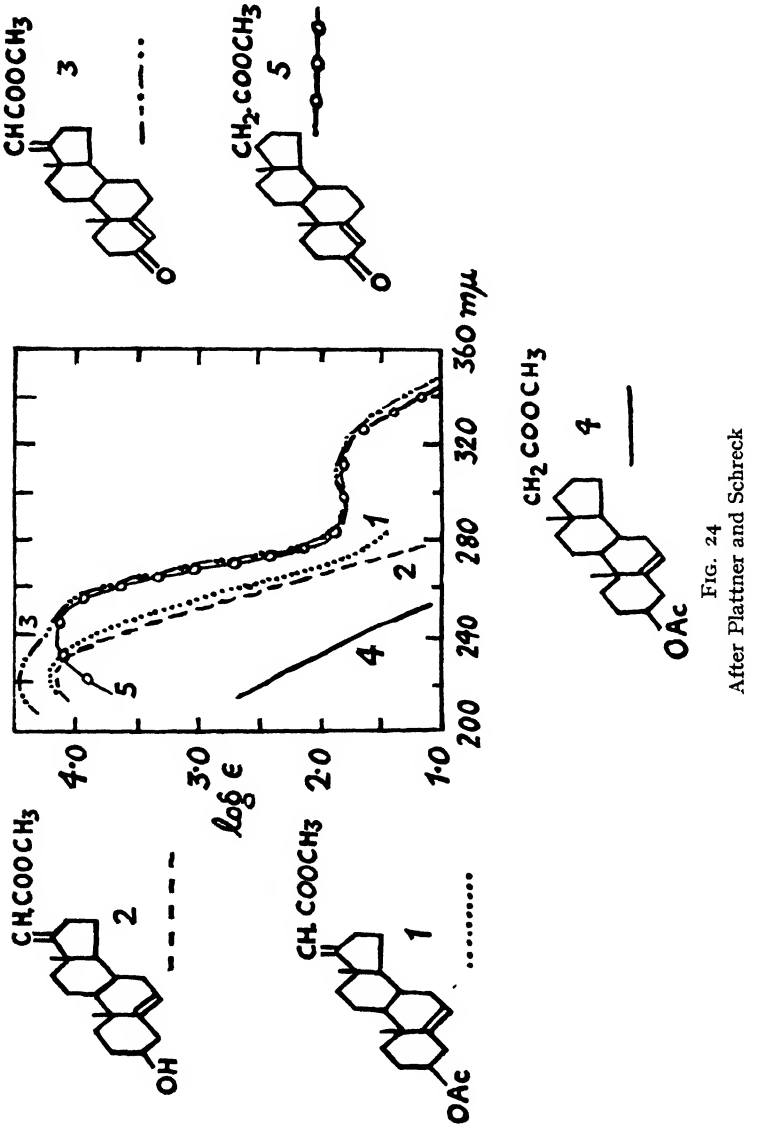


FIG. 24
After Plattner and Schreck

This work on the "cortin" hormones is of great importance, but from the special point of view of this book, it illustrates the application of earlier generalisations on the relation between absorption and constitution rather than providing the basis for new generalisations. Reichstein has again and again used the spectroscopic criterion for $\alpha\beta$-unsaturated ketones. Plattner and Schreck (*Helv. Chim. Acta*, 1939, **22**, 1179) provide an interesting example of this application (see Fig. 24), and more recent issues of the same journal contain others which space does not permit us to detail.

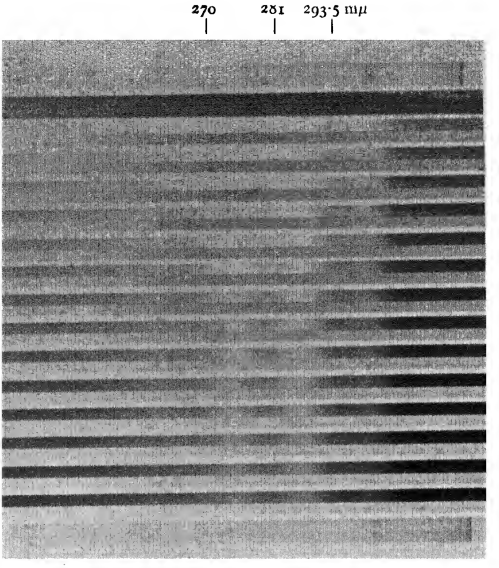


PLATE I

Ergosterol. 0·004% in alcohol, 1 cm. cell.
Under water spark between tungsten electrodes.

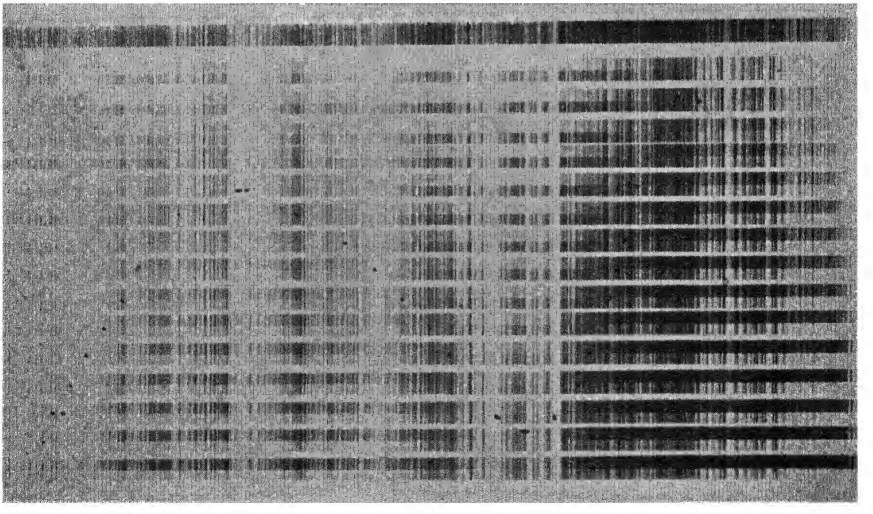


PLATE II

Absorption spectrum of vitamin A, in this case the unsaponifiable fraction from a fish liver oil moderately rich in vitamin.
2% in alcohol, 1 mm. cell. Arc between iron and nickel electrodes.

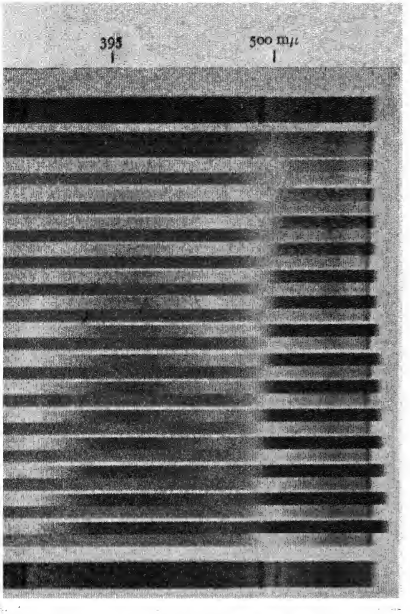


PLATE III

Absorption spectrum in the visible region of the carotene fraction from the non-saponifiable part of butter fat. Solvent: chloroform.

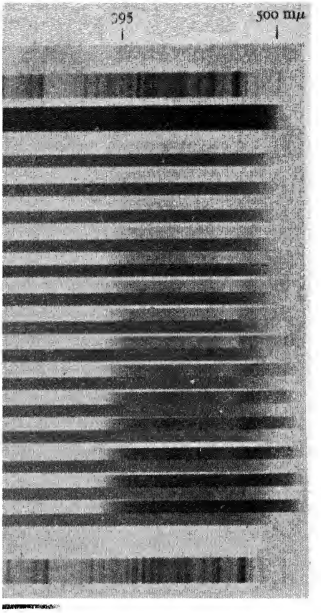


PLATE IV

Absorption spectrum in the visible region o Xanthophyll fraction from the non-saponifi able part of butter fat. Solvent: chloroform

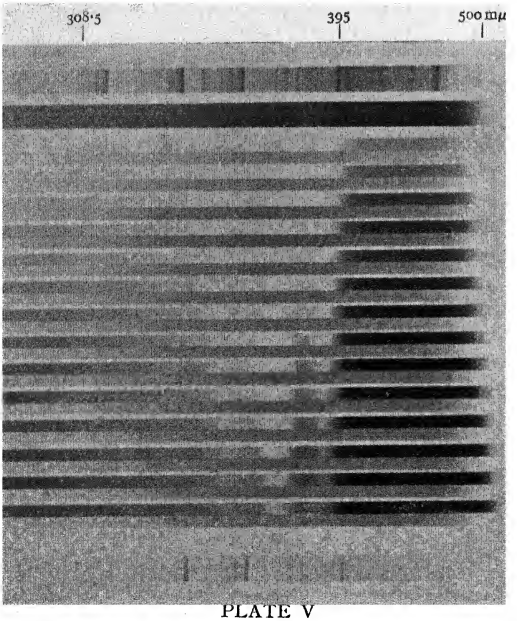


PLATE V

Cyclised vitamin A (sterol-free "non-sap" from halibut liver oil). 0·002% alcohol 8 mm. cell. Under water

CHAPTER III

PROVITAMINS AND VITAMIN A

As an introduction to the subject of provitamins and vitamin A, recent work may be summarised very briefly. The salient facts provide a framework for a more detailed discussion of the rôle played by spectroscopy in this field of research.

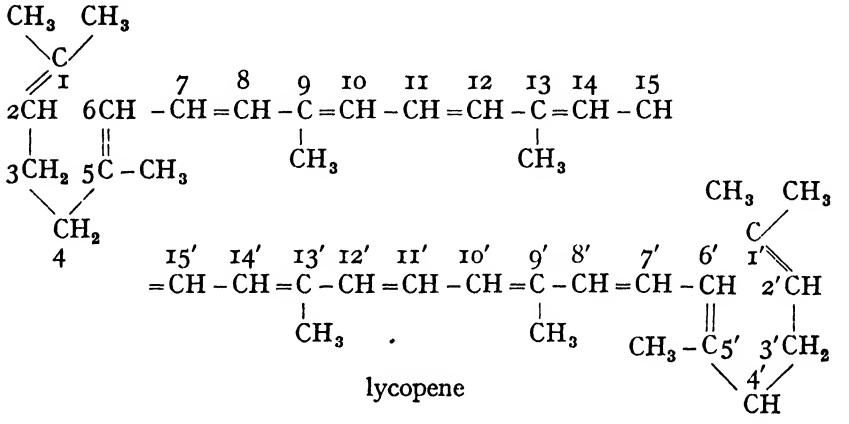
Vitamin A, $C_{20}H_{29}OH$, is a poly-ene alcohol of animal origin. It may be obtained from the livers of fishes, birds, and mammals, and from the lining of the intestines of many fishes. It is never found in vegetable products. So far as is known, it is not synthesised *ab initio* in the animal organism, but a number of precursor substances (provitamins) can be utilised by the animal and converted *in vivo* into vitamin A. These provitamins are carotenoids and all possess structures which include one half of the symmetrical β-carotene molecule, $C_{40}H_{56}$. Vitamin A_2 is closely related to vitamin A and is peculiar to fishes, especially fresh-water fishes. The A vitamins are unique in that no other vitamins are produced exclusively in animal metabolism from precursors derived (directly or indirectly) from plants only. Vitamin A has many functions, of which perhaps the best understood is its rôle in the regeneration of visual purple or rhodopsin, the retinal pigment concerned in low-intensity vision.

Carotenoids

Green vegetation contains four main pigments, chlorophyll-a, chlorophyll-b, " carotene " and " xanthophyll ". The functions of these pigments have not been properly elucidated in spite of very persistent research, and the problem of their origin and rôle in the leaf remains largely unsolved. Carotene, $C_{40}H_{56}$, is accompanied by the related dihydroxy derivative " xanthophyll ", $C_{40}H_{56}O_2$, now known as lutein. Three or more isomeric carotenes, designated α-, β- and γ-, are known, and the tomato pigment lycopene is another isomeride. These compounds belong to a large group of natural pigments soluble in fats and fat solvents. The outstanding characteristic of these *carotenoids* is the presence within the molecule of a long chain of conjugated double bonds (poly-ene chain). To this unsaturation they owe their colour and many of their most striking properties.

The simplest carotenoid is lycopene, $C_{40}H_{56}$. On complete hydro-

genation to perhydrolycopene, $C_{40}H_{82}$, 13 double bonds disappear; the corresponding fully saturated perhydrocarotene has the formula $C_{40}H_{78}$, so that carotene must contain 11 double bonds. Whilst lycopene must be a long chain aliphatic compound, carotene must possess two ring systems as well as a long chain of carbon atoms. Quantitative degradation of lycopene by (*a*) ozonisation, (*b*) permanganate oxidation, (*c*) chromic acid oxidation, led Karrer and his colleagues to establish the structure:



Ozonisation, yielding 1 mol. acetone, indicates two terminal $CH_3 \diagdown C = CH_3 \diagup C =$ groups.

$KMnO_4$ oxidation yields succinic acid, indicating $-CH_2-CH_2-$ groups, and 4 mols. acetic acid, indicating four $= C(CH_3)-CH =$ groups.

CrO_3 oxidation yields 6 mols. acetic acid, indicating two further $-C(CH_3)-$ groups.

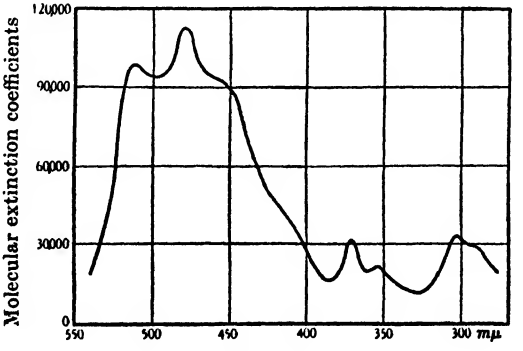


FIG. 25

Lycopene in $CHCl_3$
After Gillam and Heilbron

Lycopene shows a characteristic absorption spectrum arising from the system of 11 conjugated double bonds (see p. 58).

" Carotene " as prepared from plant products is usually heterogeneous. It yields two or more pure substances when subjected to chromatographic

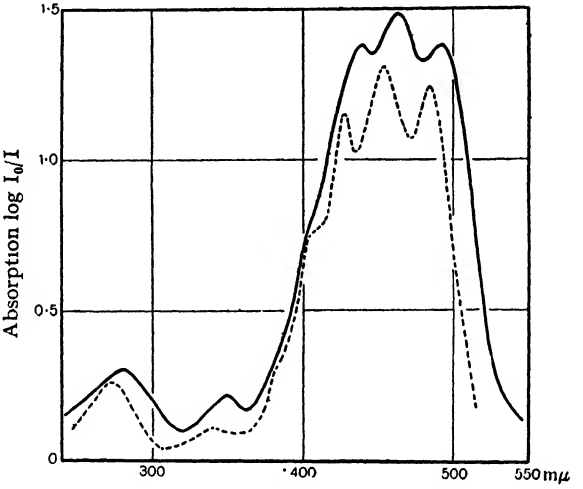
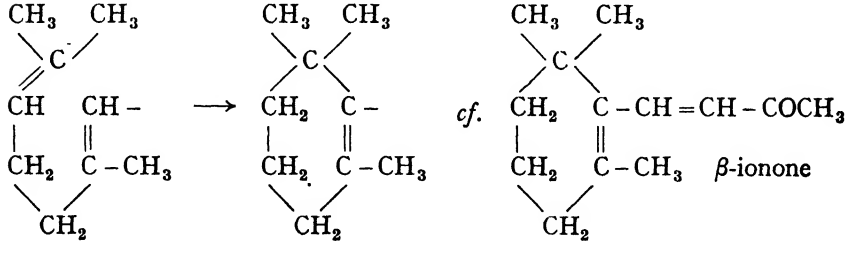


FIG. 26

——— Carotene in chloroform, 0·00078% 1 cm. cell.
– – – Xanthophyll in chloroform, 0·0042% 2mm. cell.

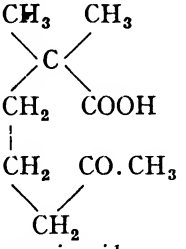
Biochem. J., 1933, **27**, p. 882.

analysis (see p. 59). The isomeric α- and β- and γ- carotenes have been degraded by oxidative methods. In γ-carotene one end of the lycopene molecule has been replaced by the cyclised equivalent:



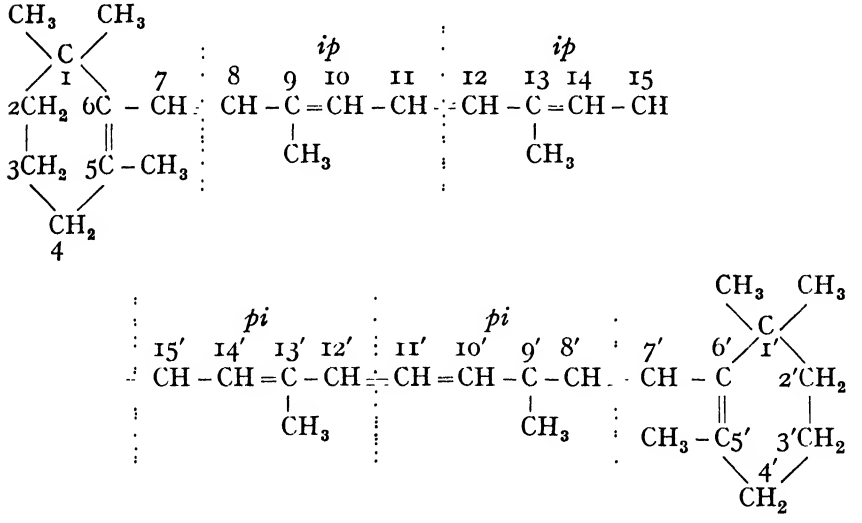
In β-carotene both ends of the lycopene molecule have been replaced by β-ionone rings, whilst α-carotene is unsymmetrical, containing one terminal β-ionone ring and one terminal α-ionone ring. On ozonisation

β-carotene yields geronic acid in approximately the expected amount for a β-ionone derivative (Karrer, Helfenstein, Wehrli and Wettstein, *Helv. chim. Acta*, 1930, **13**, 1084).



geronic acid.

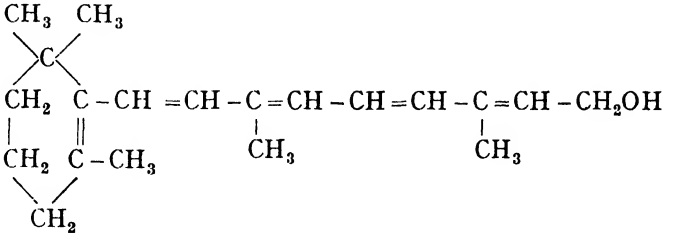
The long chain common to the $C_{40}H_{56}$ isomerides is made up of four isoprene units arranged in pairs which are united in reverse order at the carbon atoms 15 and 15'.



β-carotene

A large number of carotenoids have been tested biologically for provitamin A activity. The only active ones are those which contain one-half of the symmetrical β-carotene molecule unchanged. In nearly every case the potency of provitamins A other than β-carotene is about one-half that of the β-isomeride; α- and γ-carotene, kryptoxanthin, aphanin and aphanicin (see p. 56) are active. Myxoxanthin and echinonene are probably also provitamins A.

When Karrer, Morf and Schöpp (*Helv. Chim. Acta*, 1931, **14**, 1431) suggested the formula



on the basis of degradations carried out on very rich vitamin A concentrates from fish liver oils, the structure was in harmony with that of β-carotene, and it seemed as if the fission achieved within the animal

$$\underset{(\beta\text{-carotene})}{C_{40}H_{56}} \xrightarrow[2H_2O]{\textit{in vivo}} 2C_{20}H_{29}OH \quad \text{(hypothetical) or}$$

$$\underset{\text{(other provitamins)}}{C_{20}H_{28}X} \xrightarrow[2H_2O]{\textit{in vivo}} C_{20}H_{29}OH + XHOH$$

amounted to adding the elements of water at C_{15} or $C_{15'}$. The experimental animal suffering from avitaminosis-A effects the transformation with high efficiency, whereas the well-nourished animal receiving carotene in excess of the minimum required fully to meet its need, rejects or

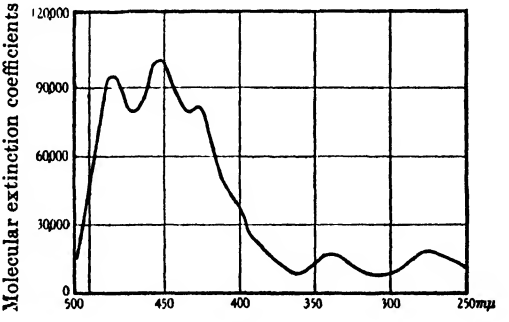
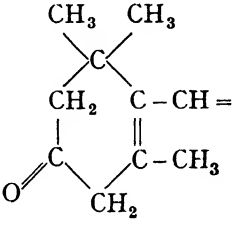


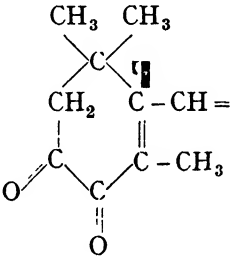
FIG. 27
Lutein in $CHCl_3$
After Gillam and Heilbron

destroys much carotene. Some claims have been advanced that the conversion, *β-carotene → vitamin A*, can be realised *in vitro*, but as yet they are unconfirmed.

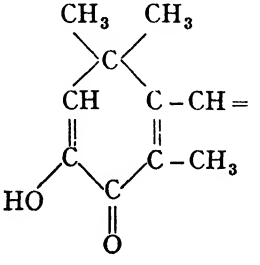
In addition to the hydrocarbon carotenoids many oxygenated "xanthophylls" occur in nature. Leaf xanthophyll or lutein is a 3, 3'-dihydroxy-α-carotene showing a spectrum indistinguishable from that of α-carotene itself. The β-carotene analogue, zeaxanthin, occurs with lutein in egg yolk. Kryptoxanthin is the monohydroxy derivative of β-carotene, and rubixanthin of γ-carotene. Lycoxanthin and lycophyll are respectively mono- and dihydroxy derivatives of lycopene. Aphanin is a 3-keto-β-carotene, one β-ionone ring being replaced by the group



Astacene (see p. 81) is a 3, 4, 3', 4'-tetraketo-β-carotene, the terminal groups being :



and it can act as an acid in the enolic form



Aphanin is a recent addition to the list of provitamins A. Tischer (*Z. physiol. Chem.*, 1937, **251**, 109 ; 1939, **260**, 257) has worked on the blue alga *Aphanizomenon flos aquae*. Heilbron and Lythgoe (*J.C.S.*, 1936, 1376) had previously studied the blue alga *Oscillatoria rubrescens* and in

addition to β-carotene and lutein had isolated two new polyene pigments, myxoxanthin (Chart V) and myxoxanthophyll, $C_{40}H_{56}O_7$ (±2H), *m.p.* 169°:

$\lambda_{max.}$ 518, 484·5 450 mμ ($CHCl_3$)
$\lambda_{max.}$ 503, 471, 445 mμ (CH_3OH)

Tischer found in *Aphanizomenon* neither of the above, but succeeded in isolating four new carotenoids:

		yield on dry algae	*m.p.*	$\lambda_{max.}$ mμ CS_2	petrol-ether	CH_3OH
aphanin	$C_{40}H_{54}O$	50mg./kg.	176°	533	494	491·5
				494	460	457
					432	
aphanicin	$C_{80}H_{106}O_3$	20mg./kg.	190°–195°	533	494	491·5
				494	462	457
flavacin			155°	490	458	
				457	428	
				424		
aphanizophyll		16mg./kg.	172°–173°	523		507
				487·5	($CHCl_3$)	475
				457		444
β-carotene		100mg./kg.	184°			

Scheunert and Wagner (*ibid.*, **260**, 273) tested aphanin biologically against the β-carotene standard (see p. 86) and found it to possess the expected activity, namely about half that of β-carotene. Aphanicin appears to be a dicarotenoid derived from 2 molecules of aphanin, the two halves of the new molecule being linked by an oxygen bridge (Tischer, *loc. cit.*):

$$2C_{40}H_{54}O - 2H + O \rightarrow C_{80}H_{106}O_3$$

It also exhibits vitamin A activity, but the potency is approximately 1/4 that of β-carotene. Another addition to the list of provitamins A is leprotene, $C_{40}H_{54}$ (Takeda and Ohta, *Z. physiol. Chem.*, 1939, **258**, 7), apparently a dehydro-β-carotene:

solvent	$\lambda_{max.}$mμ
CS_2	517, 479, 447
$CHCl_3$	495, 460, 428
C_6H_{14}	484, 452, 425

The vitamin A activity is claimed to be due to the new substance itself rather than to a contaminant. It is, however, much less than that shown by β-carotene.

Three methods of study have dominated the progress of research in the carotenoid field, namely (1) partition of the crude pigment fraction between immiscible solvents such as petrol ether and 90% methyl alcohol; carotenoid hydrocarbons being, like esters, epiphasic, *i.e.* preferentially soluble in hydrocarbon solvents; whilst hydroxylated carotenoids are

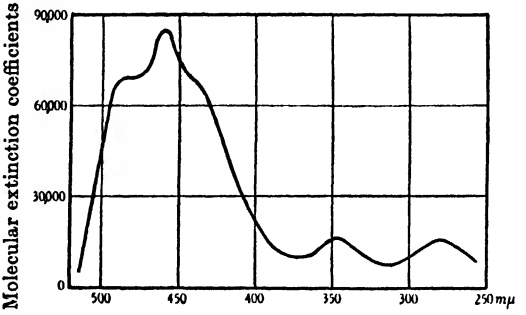
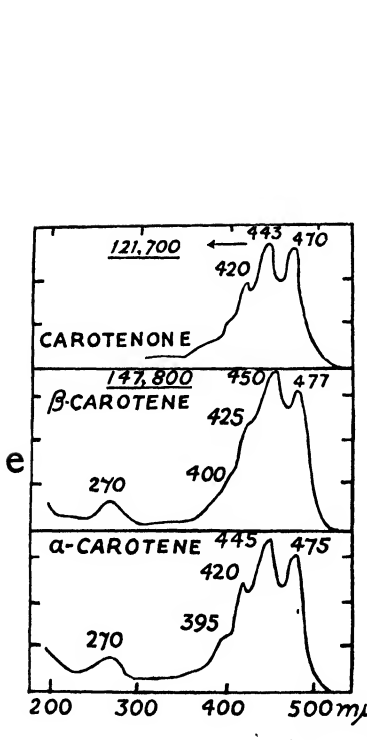


FIG. 28
Zeaxanthin in $CHCl_3$
After Gillam and Heilbron.



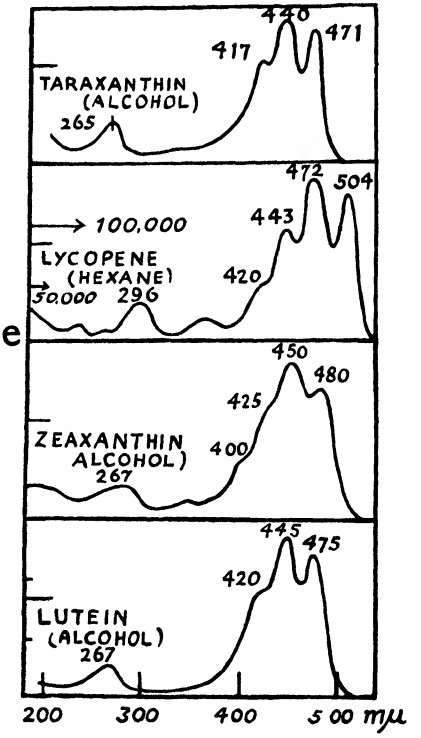


FIG. 29 (Adapted from Smakula) FIG. 30

hypophasic, *i.e.* preferentially soluble in the aqueous alcohol. (2) Spectroscopic criteria, using maxima in different solvents, afford much assistance in the task of characterising carotenoids. (3) Tswett's method of analysis by chromatographic adsorption, apart from its use by Willstätter and Stoll in their classical work on carotene, was for long neglected, but Kuhn, Lederer and Wintersteiner found in it a very delicate weapon for isolating individual carotenoids in a state of purity. The method has since acquired an extensive literature of its own and for many purposes has become indispensable (*cf.* ' Chromatographic Analysis', Zechmeister and Cholnoky, trans. Bacharach and Robinson, London, 1941).

[In addition to these three methods of study, optical rotations and micro-analytical methods have proved of great value.]

Some carotenoids are much more strongly adsorbed than others on suitably chosen solids. The two factors determining the strength of adsorption are: (*a*) the number of conjugated double bonds, (*b*) the number of hydroxyl groups, in the molecule.

TABLE IV

Adsorption of carotenoids from petrol ether solution:

			Double bonds		$\lambda_{max.}$ in CS_2	
			conjugated	unconjugated	mμ	mμ
Weakest adsorption	α-carotene		10	1	508	577
	β-carotene		11	0	518	485
Al_2O_3 as adsorbent	γ-carotene	$C_{40}H_{56}$	11	1	533	496
	lycopene		11	2	548	507
	rhodoxanthin	$C_{40}H_{56}O_3$ ketone	12 + two carbonyl groups	0	564	425
	lutein	$C_{40}H_{56}O_2$	11	1	511	478
	zeaxanthin	$C_{40}H_{56}O_2$	11	0	520	484
$CaCO_3$ as adsorbent	flavoxanthin	$C_{40}H_{56}O_3$	11	?	478	447
	taraxanthin	$C_{40}H_{56}O_4$	11	?	501	469
	violaxanthin	$C_{40}H_{56}O_4$	9	1	501	469
strongest adsorption	fucoxanthin	$C_{40}H_{56}O_6$ ketone	9 + two carbonyl groups	0	*492	457

*In $CHCl_3$

Although the carotenoids occur only in minute amounts in many natural products they are very widely distributed. The simplest vegetable organisms contain carotene and related substances, thus the Phaeophyceae (brown algae) all contain fucoxanthin and the Cyanophyceae (blue green algae) contain myxoxanthin or aphanin. In the higher plants, many of the hydroxylic carotenoids occur as esters, lutein perhaps predominating among the "xanthophylls" and β-carotene among the

hydrocarbons. Carotenoids are also found in small quantities at various sites in mammals, thus many body fats owe their yellow colour to carotenoids. Yellow bone marrow contains β-carotene, and there are small quantities in the blood and in the retina. This and other evidence shows that carotene and lutein are the characteristic animal carotenoids.

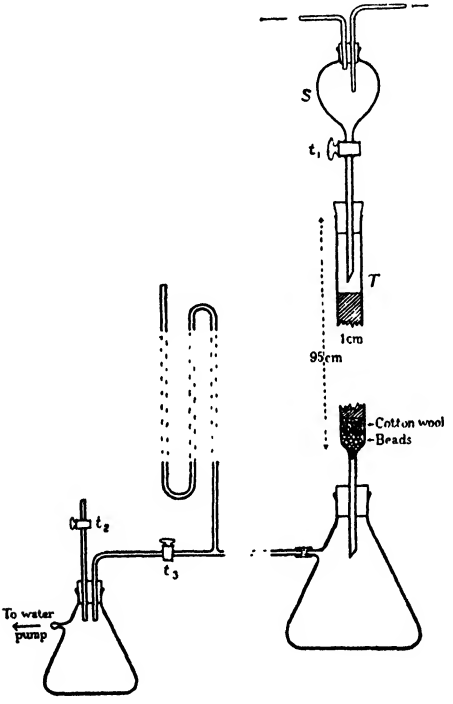


FIG 31
Apparatus for chromatographic adsorption.

This is not surprising since they are the predominating lipochrome pigments in the diet of herbivora. Our ignorance concerning the complete physiological functions of carotenoids can be shown by many unexplained observations. Thus birds accumulate "xanthophylls" rather than hydrocarbon carotenoids; horses and cattle store carotene but not lutein; in the human, both carotene and lutein are found in blood serum, whilst pigs, cats, dogs and rats do not appear to store carotenoids at all. On a diet devoid of carotenoids the yolks of birds' eggs become very pale, and the plumage of canaries only remains yellow if lutein is present in the diet.

Carotenoids also occur in bacteria, and in crustaceans (see p. 80) and zooplankton generally, as complex chromoproteins. It is thought that the high vitamin A content of fish liver oils is probably due to provitamins derived from phytoplankton. The clearest evidence for this lies in seasonal fluctuations in the potency of halibut liver oil (Lovern, Edisbury and Morton; *Biochem., J.*, 1933, **27**, 1461) which show a marked

correlation with plankton activity (Fig. 32), but all attempts to show that the intake of known provitamins is sufficient to account for the vitamin A reserves have failed.

TABLE V

Sources of some carotenoids

α-carotene	red palm oil, chestnuts, carrots, berries of mountain ash
β-carotene	green leaves, carrots, red palm oil, butter
γ-carotene	fruit of Gonocaryum pyriforme (Dutch E. Indies), leaves of lily of the valley
kryptoxanthin	yellow maize, egg yolk, green grass
lutein	green leaves and grass
rhodoxanthin	seed coats of yew
astaxanthin	lobster, salmon, shrimps, etc.
lycopene	ripe tomatoes, water melon
capsanthin	red peppers, paprika, pimento
fucoxanthin	brown algae
taraxanthin	dandelions, sunflowers
violaxanthin	yellow pansies
flavoxanthin	buttercups
zeaxanthin	green leaves, egg yolk, yellow maize
myxoxanthin	blue-green algae
violerythrin	sea anemones
rhodoviolascin	purple bacteria

Vitamin A

The starting point in the study of vitamin A as a separate entity was the recognition that fish liver oils contain two accessory food factors, the specifically antirachitic vitamin D, and vitamin A which is described as "growth promoting, antixerophthalmic and anti-infective". For many years biological tests for vitamin A were exceedingly difficult to carry out quantitatively (*cf.* Coward, *The Biological Standardisation of Vitamins*, London, 1938). Even when assays were far from being precise, it was possible, by supplementing the diet of experimental animals depleted of vitamin A, to obtain a quick resumption of growth (measured by weight increase). The early workers observed a suggestive parallelism in foodstuffs of vegetable origin between carotenoid concentration and vitamin A potency, thus yellow maize was repeatedly found to be superior to the pale variety.

The biological method of control afforded proof that the vitamin A of cod liver oil passes quantitatively into the non-saponifiable fraction, from which the bulk of the sterols can be removed by freezing at −60°,

using methyl alcohol as solvent (McCollum and Davis, *J. Biol. Chem.*, 1914, **19**, 245 ; Steenbock and Boutwell, *ibid.*, 1920, **58**, 14 ; Drummond and Coward, *Lancet*, 1921, ii, 698).

Rosenheim and Drummond (*Biochem. J.*, 1925, **19**, 753) observed that liver oils and extracts gave a brilliant blue colour with arsenic trichloride, the intensity varying apparently with the vitamin content. Carr and Price (*Biochem. J.*, 1926, **20**, 497) found that a saturated solution of antimony trichloride in chloroform was more convenient and answered

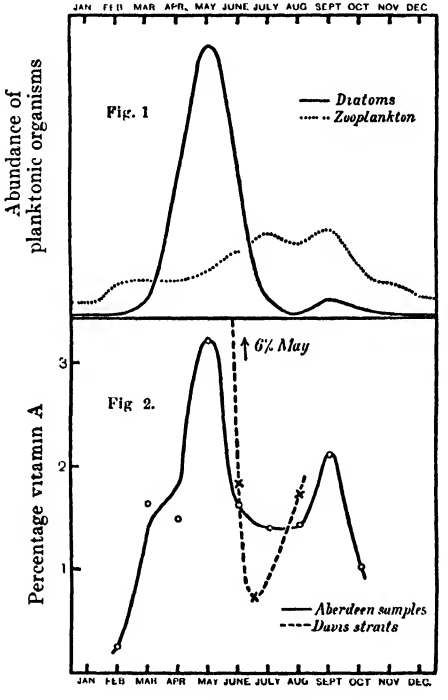


FIG. 32

1.—Mean annual plankton variations, 1907-20, Port Erin Bay ; adapted from Johnstone *et al.* [1924].

The diatom curve is roughly quantitative. The zooplankton curve, which is more diagrammatic, reflects the July and September maxima due to copepods, the main curve being modified by larvae (early spring and November) and protozoa (June-July).

2.—Seasonal variations in vitamin A content of halibut-liver oils (1932).

Insufficient samples were obtained during March to justify drawing the curve with a further subsidiary maximum in the early spring.

Biochem. J., 1933, **27**, 1461.

equally well. It was very difficult, however, to prove that the chromogen was actually vitamin A, rather than a closely related substance accompanying it in the separations.

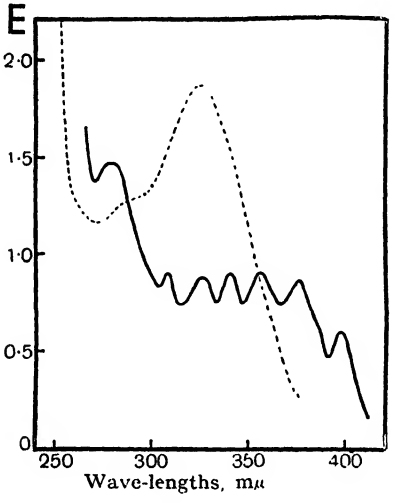


FIG. 33

– – – Cod liver oil, 1%, alcohol 1 cm.

——— 'Regenerated' oil in $CHCl_3$

Biochem. J., 1932, **26**, 1165

The term regenerated here means recovered by solvent extraction after the blue solution (with $SbCl_3$) has been poured into a large volume of water. Note the narrow bands due to cyclisation of the vitamin, (*cf.* Plate V).

Peacock (*Lancet*, 1926, ii, 328) had already shown that vitamin A was liable to decompose under the action of light, an observation which suggested that the vitamin might be able to absorb ultra-violet rays. Morton and Heilbron (*Nature*, 1928, **122**, 10; *Biochem. J.*, 1928, **22**, 987) therefore carried out quantitative studies of ultra-violet absorption and chromogenic power on a series of preparations derived mostly from fish livers. A very close correlation was established between the intensity of colour in the Carr-Price test and the intensity of an absorption band with its maximum at 328mμ (325mμ is now regarded as the maximum). Further work strengthened the belief that the 325mμ band was due to vitamin A itself. Thus the photochemical destruction of vitamin A was achieved by exposing the material in quartz vessels which had been lightly silvered on the outside, the success of the experiment depending upon the fact that the transmission spectrum of thin layers of silver corresponds very closely with the absorption spectrum ascribed to vitamin A. As the irradiation went on, the ultra-violet absorption decreased at about the same

rate as " chromogenic power " towards antimony trichloride, and feeding tests gave rough confirmation of a decrease in vitamin content. Comparison of physical tests with biological activity (Drummond and Morton, *Biochem. J.*, 1929, **23**, 785; Coward, Dyer, Morton and Gaddum, *Biochem. J.*, 1931, **25**, 1102; 1932, **26**, 1593) provided more quantitative evidence for the 325mμ absorption band as a property of vitamin A.

Cod liver oil has long been available in quantity, partly because immense catches of cod are made and partly because the high oil content of the livers permits " extraction " by simple cooking in steamers. The vitamin A content is, however, only very moderate, and it is now known that the oil is a much less suitable starting material for the isolation of vitamin A than certain other fish liver oils, *e.g.* those of halibut, tunny,

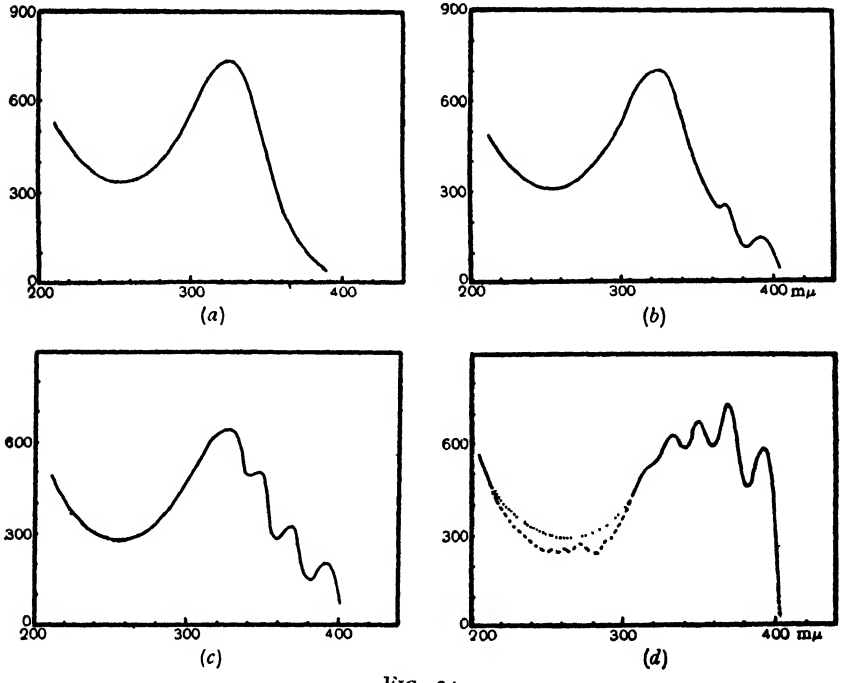


FIG. 34

Absorption curves illustrating successive stages in the action of alcoholic hydrogen chloride on vitamin A.

(*a*) Vitamin A concentrate in absolute alcohol.

(*b*) The same concentrate in alcoholic hydrogen chloride showing the earliest detectable change.

(*c*) A later stage in which the narrow bands are more clearly shown.

(*d*) The absorption curve recorded when the narrow bands have attained maximum definition. The broken portion of the curve is not very reproducible.

Biochem. J., 1932, **26**, 1168

soupfin-shark and sturgeon. Although there may not be a great deal of fat in all such livers, the vitamin A content is often exceedingly high. It is customary to put the livers into cold storage on board ship and to work them up in shore factories.

Saponification of such rich fish liver oils yields highly potent non-saponifiable fractions (*cf.* Takahashi and Kawakami, *J. Chem. Soc., Japan*, 1923, **44**, 380, 590). Freezing at $-60°$ eliminates most of the cholesterol, and selective adsorption, using a column packed with alumina specially prepared according to Brockmann's directions,* permits still further concentration. Distillation at very low pressure, using a molecular still (see p. 66), allows an even higher degree of purification. If the distillation is carried out at somewhat higher pressures, vitamin A undergoes cyclisation. Heilbron, Morton and Webster (*Biochem. J.*, 1932, **26**, 1194) obtained from the cyclised product a naphthalene compound, the formation of which from vitamin A could only be accounted for by accepting Karrer's formula (p. 55). Karrer, Morf and Schöpp (*Helv. Chim. Acta*, 1933, **16**, 557) clinched the matter by synthesizing crystalline perhydrovitamin A (the fully hydrogenated derivative of vitamin A) by a long sequence of reactions (8 steps) starting with β-ionone. The fully hydrogenated natural vitamin A from fish liver oil proved identical with the synthetic product.

For several years failure met all attempts to induce vitamin A to crystallise, although all the evidence went to show that the richest preparations were not far from pure. This failure was important because definite criteria of purity could scarcely be obtained for a " pale yellow, highly viscous oil " of questionable stability. A similar lack of success awaited many attempts to prepare crystalline esters until Hamano (*Sci. Papers Inst. Phys. Chem. Res., Tokyo*, 1935, **28**, 44, 69 ; 1937, **32**, 44) obtained the β-naphthoate and the anthraquinone carboxylate. Holmes and Corbet (*Science*, 1937, **85**, 103 ; *J. Amer. Chem. Soc.*, 1937, **59**, 2042) achieved a considerable success in preparing crystalline vitamin A from a rich fish liver oil (ishinagi oil) :

KOH (14g.) ground roughly, is warmed with pure isopropyl alcohol (103 c.c.) under reflux. The solution is decanted (from any carbonate) on to 25g. of oil. Gentle agitation for 1½ hrs. results in saponification. The soap jelly is dissolved in 150 c.c. boiled water, and 265 c.c. water and 530 c c. petrol ether are added while the flask is rotated gently. 100 c.c. of 95% alcohol are then added. The petrol ether layer is removed and 4 more extractions are made, using 500 c.c. of petrol ether each time. The combined extracts are reduced to 40–50 c.c. on the water bath, and the " unsaponifiable " is transferred to crystallising dishes and reduced to dryness in a vacuum desiccator. The residue is dissolved in methyl alcohol (0·06–0·08g./c.c.). Much sterol can be removed by filtration after cooling to $-5°$ C , and the filtrate is placed in a N_2-filled bottle which is left in contact with solid CO_2, and it may be necessary to filter again at a low temperature. The liquid is reduced to half bulk and a further crop of crystals obtained. " A vital step at this stage is the addition

* See Zechmeister and Cholnoky, *op. cit.*

of about 1% of water before returning the filtrate to the refrigerator. If no precipitation occurs in a few days' time another 1% of water is added without stirring, since the crystalline nuclei forced out by excess water at the top of the solution aid greatly in the growth of a larger mass of crude vitamin A." The crude crystalline mass may be recrystallised from pure methyl alcohol in an atmosphere of N_2 with occasional addition of a few drops of water to start crystallisation. The crystallising vessel is kept in the solid CO_2 refrigerator. The vitamin forms pale yellow needles, *m.p.* 7·5–8·5° C. It analyses correctly for $C_{20}H_{29}OH$* and is optically inactive. The ultraviolet absorption occurs in the expected position, $\lambda_{max.}$ 325mμ. The extinction coefficient appeared to be $\epsilon_{max.}$ 60,000 ($E^{1\%}_{1cm.}$ 2100) on the freshly diluted solution.

The antimony trichloride colour test was somewhat variable (80,000–100,000). The biological value was 3×10^6(±20%) I.U./g.

Subsequent experience has shown that the crystals were solvated. Pure vitamin A alcohol, solvent free crystals, has *m.p. ca* 60° C. and $E^{1\%}_{1cm.}$ 325mμ *ca* 1880.

Hickman (*Ind. Eng. Chem.*, 1937, **29**, 968) has applied the process of molecular distillation to rich oils and concentrates with great success (see also p. 94). Very rich concentrates prepared in this way by Mead (*Biochem. J.*, 1939, **33**, 589) were used for the preparation of several esters, of which the β-naphthoate and anthraquinone β-carboxylate were crystallised.

VITAMIN A ESTERS

	β-naphthoate	anthraquinone β-carboxylate	
m.p.	78°	126° yellow form	118°–120° (less stable) red form
$E^{1\%}_{1cm.}$ 325mμ	1185	938	1090

(see also p. 95)

The principal remaining problem in the chemical study of vitamin A is the difficult one of synthesis. A series of papers by Heilbron and his colleagues (*J. Chem. Soc.*, 1935, 585; 1936, 561; 1937, 755; 1938, 178) contains important steps in this direction. Claims to have achieved the synthesis (Fuson and Christ, *Science*, 1936, **84**, 294; Kuhn and Morris, *Ber.*, 1937, **70B**, 853) are unconvincing and can hardly be accepted on the evidence disclosed.

At a much earlier stage in the development of vitamin A studies it was established that supplements of vegetable products prevented the symptoms of deficiency from appearing. The very reasonable assumption that the vitamin A response was always due to one and the same accessory food factor proved difficult to reconcile with the failure to detect the

* This misled the authors (see below).

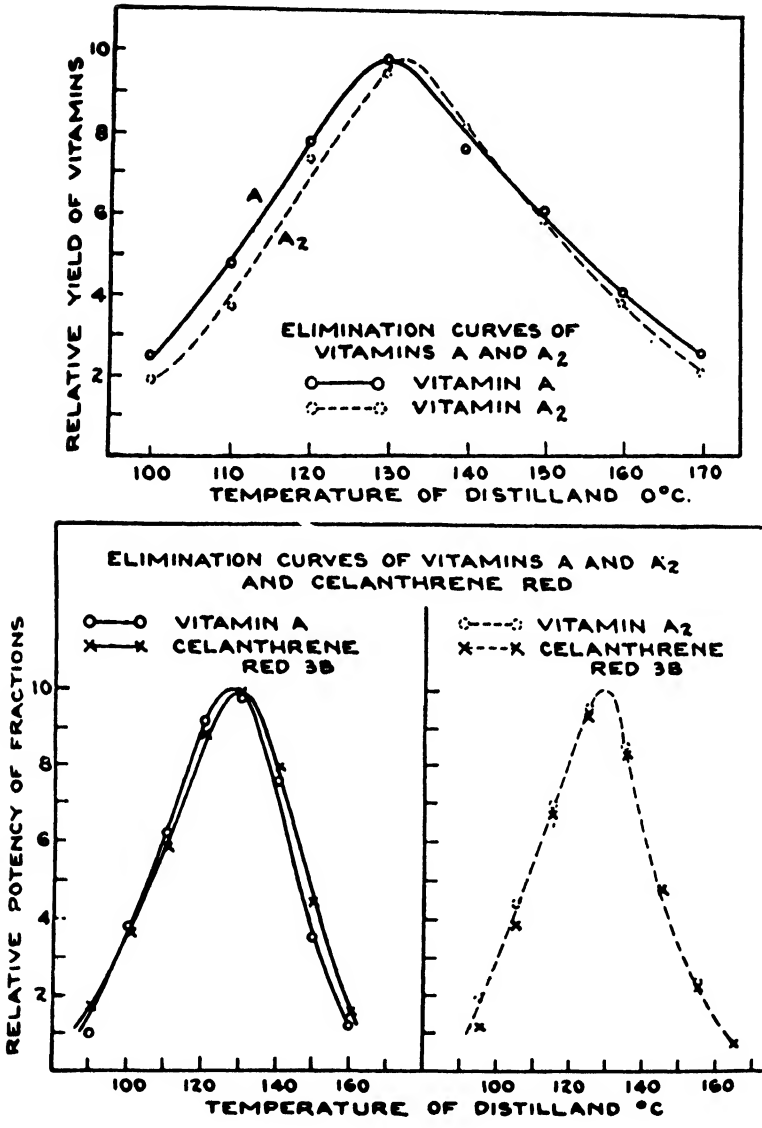


FIG. 35

After Hickman

These curves illustrate Hickman's work on molecular distillation and show the close similarity shown by vitamins A and A_2.

The dyestuff, if added in very small amount will be eliminated with the vitamins and the depth of colour shown by a distillate fraction provides a good indication of the potency, because the elimination curves of vitamin and dye are so nearly identical.

" fish oil vitamin A " in plant products, although the potency should have made such recognition possible. Euler (*Biochem. Z.*, 1928, **203**, 370; *Helv. Chim. Acta*, 1928, **12**, 278), following up hints scattered in the literature, settled the matter by showing that " pure " carotene at very low doses gave exactly the same response as fish liver oils when administered to animals lacking vitamin A. The vitamin A of fish liver oils was known, however, to be almost colourless and it was easy to demonstrate spectroscopically that the richest vitamin A concentrates contained neither carotene nor other carotenoids (Drummond, Ahmad, and Morton, *J.S.C.I.*, 1930, **49T.**, 291). The penetrating work of Moore (*Lancet*,

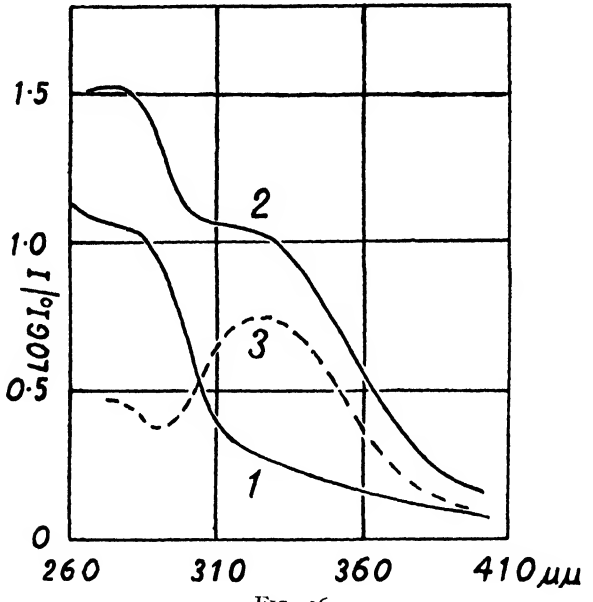


FIG. 36

1. Rat liver fat (low carotene diet).
2. Rat liver fat (high carotene diet).
3. Curve 2 minus curve 1. (0·97% alcoholic solution 4 mm. thickness).

After Drummond, Ahmad and Morton

1929, 499; *Biochem. J.*, 1929, **23**, 803, 1267; 1930, **24**, 696; 1931, **25**, 275) resolved the dilemma by proving that addition of plentiful supplies of carotene to vitamin A-free diets brought about a substantial increase in the amount of vitamin A (recognised by its ultra-violet absorption) stored in the livers of experimental animals. This conversion of carotene to vitamin A in the organism has since received ample confirmation, and indeed pure β-carotene has been adopted as the International Standard of vitamin A activity (see p. 86). Olcott and McCann (*J. Biol. Chem.*, 1931, **94**, 185) have suggested that the change *carotene* ⟶ *vitamin A* is due to

the action of a specific enzyme, *carotenase*, present in liver tissue and have also claimed that the conversion could be realised *in vitro* by incubating carotene with extracts of minced liver. Other observers, using spectroscopic methods, have failed to confirm these observations (*cf.* Euler and Klussmann, *Erg. der Physiol.*, 1932, **34**, 360; Rea and Drummond, *Z. Vitaminforsch.*, 1932, **1**, 177). Not only have repeated attempts (both published and unpublished) to transform carotene into vitamin A *in vitro* failed, but, in addition, introduction of colloidal carotene into the livers of living cats by perfusion via the splenic vein led to no detectable increase in absorption at 325mμ in extracts subsequently obtained from the liver.

Furthermore, the fate of carotene administered orally has been followed through the alimentary canal to the faeces, without appreciable light being thrown on either the site or the mechanism of conversion. Euler has suggested, albeit somewhat vaguely, that the transformation may occur in the blood stream. His observation that both vitamin A and carotene occur in blood is a fact the full significance of which remains tantalisingly obscure (see p. 100).

The physiology of carotenoids and of vitamin A

In studying the occurrence, utilisation and storage of carotenoids it is necessary to use spectroscopic methods of analysis or colorimetric alternatives. Biological assays, quite apart from being costly and time-consuming, do not discriminate between carotenoid provitamins and the vitamin itself; moreover, they only afford quantitative data at dose-levels near to the minimum needed to relieve deficiency symptoms. Biological methods of experimentation are of course fundamental, but there must be a constant interplay between physico-chemical assays and bioassays if either technique is to make the full contribution of which it is capable.

The exact rôle of carotene in the plant economy is unknown; the carotenoids appear when growth is active and a suggestive parallelism has been noted between carbon dioxide assimilation through chlorophyll, and carotenoid concentration. Green shoots are very much richer in carotene than etiolated shoots, a fact which may imply that carotene is itself a product of photosynthesis. There is no diminution in carotene content during periods of darkness, and the pigment persists after the leaves have turned colour. Dead leaves contain no carotene, but freshly cut and artificially dried grass or lucerne, retains much unoxidised carotene. This conservation of provitamin A is of great practical importance (see p. 101). If cattle or fowls are provided in the winter with a diet containing carotene, the provitamin and vitamin A content of milk (and butter) or eggs may remain near the optimum level associated with a summer diet rich

in green foodstuffs. This in turn provides provitamins—and vitamin A —for human nutrition.

There is no certainty that carotene plays any part in the animal economy other than that of a provitamin A. It would, however, be unwise to assume because carotene is so important as a vitamin A precursor that it lacks functions of its own. There is apparently a normal blood level in respect of carotene concentration, and in many species storage takes place in adipose tissue. In other species, carotene does not appear to be retained as such. The pigment of yellow bone marrow from cattle is almost entirely β-carotene and it is not accompanied by vitamin A (little is known about bone marrow from other animals). The fact that carotene appears in eggs and milk is in itself suggestive of a function or functions characteristic of the hydrocarbon itself. There is no evidence that the conversion of carotene to vitamin A *in vivo* is a reversible process, but it would be very interesting to feed experimental animals on a diet free from carotenoids but rich in vitamin A and to examine the bone marrow for carotene.

The animal body in health contains only small quantities of carotene and is not equipped to assimilate large doses. Excessive intake results in some destruction, probably in the intestine, and the faeces very frequently contain large quantities of apparently unchanged carotene. In the blood, carotene reaches a maximum some 8 hours after ingestion, whereas the vitamin A content reaches its maximum some 3–5 hours after administration of the vitamin A as such. Carotene assimilation is optimal, possibly as much as 80%, when minimal doses are fed in fat, but if liquid paraffin is used as solvent there is a marked decrease in "utilisation", *i.e.* assimilation plus destruction, at both low and high dose-levels. It appears that the transport of carotene through the intestinal wall is conditional on normal fat absorption (Clausen, *J. Amer. Med. Ass.*, 1933, **101**, 1384; Heymann, *Am. J. Dis. Child.*, 1936, **51**, 273; Ahmad, *Biochem. J.*, 1931, **25**, 1195). This is confirmed by the failure to deal with carotene in jaundice, or by choledochocolonostomised dogs (Greaves and Schmidt, *Am. J. Physiol.*, 1935, **111**, 492, 502). Bile acids appear to play a part in the transfer of carotene through intestinal epithelia. The site of conversion of carotene into vitamin A is generally held to be the liver although the evidence falls short of proof. In most species, carotene finds its way into the blood stream and soon afterwards granules of pigment may, in certain circumstances, be found in the Kupffer cells. There seems to be a temporary accumulation of carotene in the liver followed by a rise in vitamin A content (Drummond). Phosphorus poisoning results in a decrease in vitamin A formation. Perhaps, however, the most direct evidence is that cattle suffering from diseases affecting the parenchymal cells in the liver accumulate large quantities of carotene in the Kupffer cells. In diabetes,

the liver reserves are very high and carotenemia is common, indicating perhaps defective metabolism of both preformed vitamin A and provitamin A.

Vitamin A in milk

The vitamin A activity of milk is important not only because of the essential part played by this food in the diet of the very young, but also because of the very low *reserves* of vitamin possessed by the newborn (Dann, *Biochem. J.*, 1934, **28**, 2141). A certain amount of unchanged carotene is always transmitted in the milk. The relative proportions of carotene and vitamin A vary with the species and the breed, although under similar conditions the *total* is of the same order in the different breeds.

Proportion of vitamin A activity

	due to carotene	due to vitamin A
Guernsey Milk	2/3	1/3
Ayrshire ,,	1/3	2/3
Jersey ,,	½	½
Goat ,,	negligible	nearly all

(Baumann, Steenbock, *et al.*, *J. Biol. Chem.*, 1934, **105**, 167).*

There is considerable evidence that most species have their own characteristic level of vitamin A potency for normal milk. This level is not strictly maintained, but it fluctuates over a small range only. An exception must be made for colostrum, which may contain 10–100 times as much vitamin A as the later milk (Dann, *Biochem. J.*, 1936, **30**, 1644). This enables the newborn calf to build up a reserve. Dann has also shown that human colostrum is 2–5 times as potent as early milk, which is itself 5–10 times as rich as ordinary cows' milk. Late human milk resembles ordinary cows' milk in both vitamin A and carotene content.

Eggs are subject to similar variations. Pale egg yolks normally indicate a diet low in carotenoids and vitamin A. The total A activity of pale yolks, however, is normal if cod liver oil (*i.e.* preformed A) is used in feeding the hens. If the diet is deficient in carotene or vitamin A or both, the potency of the eggs diminishes and the number laid falls off. With an excess of dietary vitamin A the potency of individual eggs will certainly increase, but if the supply is discontinued, the eggs become normal long before the unusually high liver reserves have been used up.

The earliest sign of sub-optimal vitamin A nutrition is partial night blindness, a condition which in many subjects (both human and animal) responds quickly to vitamin A therapy. Elegant work by Wald and others has greatly clarified knowledge concerning the rôle of vitamin A

in eye tissues. The pigmented layers (pigment epithelium and choroid layer) contain an esterified xanthophyll (probably lutein) and vitamin A :

Carotenoid	Vitamin A
accompanies the hydrocarbon in the phase separations, but after saponification becomes hypophasic :	epiphasic, but becomes hypophasic on saponification :
$\lambda_{max.}$ 445, 476, 504mμ (CS_2)	$\lambda_{max.}$ 325mμ
428, 456, 485 ($CHCl_3$)	(617mμ with $SbCl_3$)
450 p.p.m. dry tissue from frogs' eyes	1800 p p.m. dry tissue from frogs' eyes

In the retina, the light-sensitive elements are the rods and cones. The cones are adapted to the perception of light of high intensity and to colour discrimination, and there is evidence (Hecht) that vitamin A may be concerned. The rods contain visual purple or rhodopsin, a photosensitive pigment which initiates nerve impulses as it decomposes ; it is concerned in the perception of dim light irrespective of colour, and faulty dark adaptation is due to delayed regeneration of rhodopsin.

Boll (1876) was the first to discover visual purple and Kuhne (1877) to isolate it. The latter's work (Hermann's *Handbuch der Physiologie*, Leipzig, 1879, **3**, pt. 1, 235) was far ahead of its time and has gained the admiration of present-day authorities like Hecht. Visual purple is now known to be a conjugated protein with a carotenoid prosthetic group, and, as would be expected, it is non-diffusible and can be salted out from aqueous solution. Although it yields no colour to homopolar solvents, it is readily broken down by protein coagulants (acetone, alcohol, acids, akalies, etc.), whereupon the pigment becomes soluble in petrol. Visual purple exhibits a broad absorption band, $\lambda_{max.}$ 500mμ, and the curve relating the response of the human dark-adapted eye to different wave-lengths affords direct proof that the pigment is connected with low-intensity vision.

Wald collected 2000 ox retinas in 95% alcohol and to the solid portion he added 800g. Na_2SO_4 and extracted repeatedly with benzine. The alcoholic liquor was reduced to small bulk and extracted similarly. The combined extracts were evaporated and taken up in CH_3OH from which sterol was removed by freezing (−80°, solid CO_2). The residual retinal "oil" contained 0·2% vitamin A.

Frog retinas possessed 1/17 of the weight of ox retinas and contained more vitamin A, and the frog pigmented layers were even richer. Weight for weight, frog retinas contain 35 times as much vitamin A as ox livers.

Estimated vitamin A content of a single frog retina 400μg.
" " " " ox retina 22μg.

In his experiments on dark-adapted retinas dissected out in a feeble red light, Wald found that :

(*a*) CS_2 (or petrol) extracts vitamin A, but not visual purple.

(*b*) $CHCl_3$ denatures visual purple and decolorises it at once. After some difficulty a greenish-yellow pigment is dissolved by the chloroform. This compound, *retinene*, shows an inflexion near 405mμ and absorption maxima at 310 and 280mμ. It gives a blue colour with $SbCl_3$ (sharp band $\lambda_{max.}$ 664mμ). Retinene is freely soluble in petrol, once it has been extracted. It is therefore found in the retina as part of a non-lipoid complex.

(*c*) On exposure to daylight the retinas change colour from purple to orange and become colourless within an hour. On extraction with benzine at the orange stage, retinene at once goes into solution ($SbCl_3$, colour test 664mμ), whereas the colourless retinas yield vitamin A to the solvent ($SbCl_3$, colour test $\lambda_{max.}$ 617mμ).

(*d*) Visual purple cannot be regenerated from isolated colourless retinas, but the therapeutic value of vitamin A in night blindness indicates that regeneration must occur *in vivo*.

(*e*) As there are no blood vessels in the frog retina, vitamin A must enter by diffusion, probably from the ample reserves in the pigmented layers.

The pathological changes which result from vitamin A deficiency are ably reviewed by Bessey and Wolbach (*J. Amer. Med. Ass.*, 1938, **110**, 2077).

Vitamin A requirements

Wolbach (*Science*, 1937, **86**, 569) suggests that " vitamin A is a structural material which may be solely concerned in maintaining an apparatus within cells, and not in the chemical processes proper for which the apparatus is necessary ". The evidence does not compel assent to this interesting suggestion, but it does seem to imply a definite relationship between body weight and vitamin A needs. One method of assessing the human requirement involves the recognition of partial night blindness in a large number of subjects, the diets of whom are accurately known. This involves extensive dietary surveys and careful analysis by spectroscopic or colorimetric methods of all the food constituents. When the clinical and analytical data are compared, there should be a statistically significant correlation between defective low-intensity vision and inadequate vitamin A supplies. This field of research is summarised in recent volumes of the *Annual Review of Biochemistry*.

The chemistry and kinetics of dark-adaptation have been studied for many years by Hecht who reviews the subject in an important paper (*Physiol. Rev.*, 1937, **17**, 239). Several experimental procedures have been used for measuring dark-adaptation, and, although there is room for further research, the main findings are incontestable (*cf.* Jeans and Zentmire, *J. Amer. Med. Ass.*, 1934, **102**, 892 ; 1936, **106**, 996 ; 1937, **108**, 451 ; Jeghers, *ibid.*, 1937, **109**, 756 ; Maitra and Harris, *Lancet*, 1937, **2**, 1009; Corlette *et al.*, *Am. J. M. Sc.*, 1938, **54**, 195; Feldman, *Arch. Ophth.*, 1934, **12**, 81). The Birch-Hirschfeld photometer, modified in the newer " biophotometer ", has been widely used. In England, Maitra and Harris (*loc. cit.*) found that more than a third of the children tested from public elementary schools were below normal with respect to low-intensity vision, whereas only one boy, out of a group of forty attending a school where fish liver oil was given as a prophylactic agent, failed to pass the test. It is true that the latter group had probably been well-nourished from birth, but the substantial improvement in dark-adaptation which

occurred with vitamin A therapy in the case of the first group, seems to clinch the argument.

The daily requirement of vitamin A is not easy to determine and any figure must be accepted with reserve. The League of Nations Commission (1937) offered an estimate of 3000 I.U. More recent work stresses the fact that preformed vitamin A is assimilated at least twice as effectively as carotene (even when the carotene is administered in oil solution), and with ordinary foodstuffs the disparity may be greater. The most convincing experiments (Wagner, *Z. physiol. Chem.*, 1940, **264**, 153) lead to 2500 I.U./day of preformed vitamin A or 5000 I.U./day of carotene. It should be noted, however, that Wagner used *Vogan*, a German ester concentrate the potency of which seems to have been greatly overestimated by Moll and Reid (see p. 90). So far as can be judged, 1250 I.U./day of preformed A is the true requirement to be deduced from Wagner's tests. There is no doubt that babies, growing children, pregnant and, in particular, lactating women, need more, probably up to 5000 I.U./day (1 teaspoonful cod liver oil $\simeq$ 2000 I.U., 1 drop medicinal halibut liver oil $\simeq$ 1000 I.U.).

As far as preformed A is concerned (milk, butter, eggs and liver), few adults are able on an ordinary diet to obtain more than half their total need, but 1 oz. of carrots will provide about 3mg. carotene. Moore (*Post Graduate Medical Journal*, 1941, 52) points out clearly that not only are those foods which are rich in carotene cheap sources of provitamin A, but that most people depend on green vegetables or carrots more than on preformed vitamin A. It is only fair to point out that Friderichson and Edmund (*Am. J. Dis. Child.*, 1937, **53**, 89, 1179) claim to have established that children under two years of age are able to utilise carotene (in the form of carrot or spinach concentrates) about 10 times as effectively as preformed vitamin A. The work is based upon the visual purple response and needs checking both on account of its intrinsic importance and its unusual result. Guilbert and his colleagues (*J. Nutrition*, 1939, **18**, 169; 1940, **19**, 91), however, found that in order to prevent night blindness in cattle, sheep, pigs, rats and horses, either 25μg./kg. body weight of β-carotene or 6-8μg./kg. body weight of vitamin A is needed daily. (See also Braude, Foot, Henry, Kon, Thompson and Mead, *Biochem. J.*, 1941, **35**, 193.)

Moore's work (*Biochem. J.*, 1937, **31**, 155) on human livers obtained from victims of fatal accidents, indicated a reserve of about 0·1gr. vitamin A (*ca* 330,000 I.U.) in the healthy subject. This is not a very high reserve, especially when heavy demands are being made, as in lactation. Boller and Brunner (*Klin. Wochschr*, 1936, **15**, 1106) showed that considerable amounts of vitamin A appear in the urine in certain diseases. This excretion is large in pneumonia and other acute infections, and in chronic nephritis.

A recent note by Davies and Moore (*Nature*, 1941, **147**, 794) raises issues which may affect the whole discussion. In rats receiving a diet deficient in both vitamins A and E (see p. 103), the reserves of A in the liver were depleted much more rapidly than in control animals given dl-α-tocopherol. Prolonged deficiency of vitamin E led to secondary deficiency of vitamin A, indicated by lowered liver reserves and by the blanching of the teeth, which in rats has been associated with a shortage of vitamin A.

Recent work on the distribution of vitamin A in fishes (Edisbury, Morton, Simpkins and Lovern, *Biochem. J.*, 1938, **32**, 118; Lovern, Morton and Ireland, *ibid.*, 1939, **33**, 325; Lovern and Morton, *ibid.*, 330), has shown that relatively large quantities of vitamin occur in the alimentary tract, particularly in the pyloric caeca. (Some fishes and probably all mammals do not store vitamin A in the intestines.)

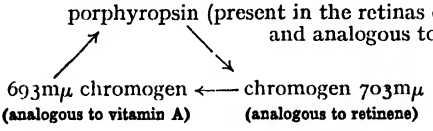
The pyloric caeca are appendages or outgrowths from the intestine, and are situated close to the pyloric extremity of the stomach and the intestinal apertures of the bile and pancreatic ducts. Absent from some species of fish, they are subject to extraordinary variations in number, size and arrangement. Many functions have been ascribed to them; fat absorption seems to be one of the most important.

Herring, cod, halibut, sturgeon, salmon and trout, all possess pyloric caeca, and these, together with the whole absorptive part of the intestine, yield oil very much richer in vitamin A than the corresponding liver oils. It is in fact likely that the practice of "gutting" fish at sea and throwing most of the pluck overboard has resulted in the loss of nearly half the available vitamin A. The vitamin A is sharply localised in the intestinal mucosa and in particular in a layer of connective tissue (the *tunica propria*) situated just below the epithelial coat. It is evident that the intestinal lipoids, which in the halibut may contain 60–70% of vitamin A esters, perform important functions in the fish. On the other hand, the intestines of mammals contain no more than traces of vitamin A. (See also, *The Chemistry and Technology of Marine Animal Oils*, with particular reference to those of Canada, *Bull.* 59, *Fisheries Res. Board of Canada*, 1941.)

Vitamin A_2

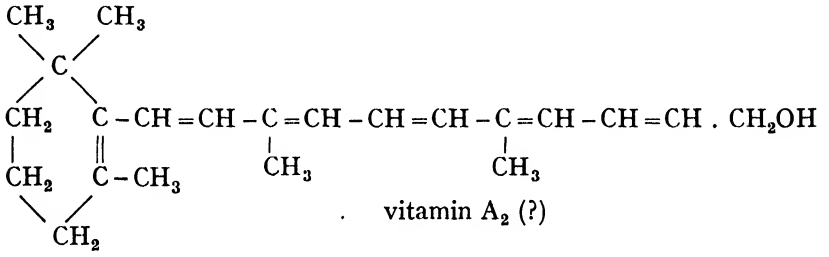
The blue solution obtained by the interaction of fish liver oils (or concentrates), and antimony trichloride, frequently exhibits absorption maxima at 690–700mμ and 635–655 mμ. Heilbron, Gillam and Morton (*Biochem. J.*, 1931, **25**, 1352), after recording these bands, postulated the existence of a chromogenic substance (or substances) other than vitamin A. The appearance of a maximum at 693mμ in a wide variety of fish liver oils indicated that vitamin A was accompanied by relatively small quantities of a congener which was not a carotenoid. For some years little progress was made in characterising the new chromogen. It accompanied vitamin

A in even the richest concentrates, no separation could be effected, and the 620mμ chromogen (vitamin A) predominated over the 693mμ chromogen to the extent of 10-20 : 1. It might have been supposed that the 693mμ band was itself due to vitamin A, had it not been observed that mammalian liver concentrates showed no evidence for the presence of the new chromogen (Heilbron, Morton, Rea, Webster and Drummond, *Biochem. J.*, 1932, **26**, 1178). This observation was recalled when Wald (*Nature*, 1937, **139**, 1017) obtained evidence for a visual cycle involving the 693mμ chromogen :



Edisbury, Morton and Simpkins (*Nature*, 1937, **140**, 234) found relatively large quantities of the 693mμ chromogen (not accompanied by comparable amounts of vitamin A) in the eyes of goldfish (*Carassius auratus*), and in the livers of trout. They therefore suggested that the 693mμ chromogen should be regarded as vitamin A_2 because its distribution suggested that it could replace vitamin A in fresh-water fish. Lederer and Rosanova (*Biochimia*, 1937, **2**, 293) had already carried out a study of liver oils obtained from fish caught in Russian rivers. Many such oils exhibited the 693mμ band in the colour test much more strongly than the 620mμ band of vitamin A, and with the non-saponifiable fractions from the best oils, only the 693mμ band could be readily seen. Such fractions when dissolved in alcohol showed an ultra-violet absorption band with $\lambda_{max.}$ 340–350mμ instead of 325mμ. This displaced absorption band was also recorded by Edisbury *et al* (*loc. cit.*). Extensions of this work have since been made (Lederer, Rosanova, Gillam and Heilbron, *Nature*, 1937, **140**, 233 ; Edisbury, Morton, Simpkins and Lovern, *Biochem. J.*, 1938, **32**, 118 ; Gillam, Heilbron, Jones and Lederer, *Biochem. J.*, 1938, **32**, 405 ; Lederer and Rathmann, *Biochem. J.*, 1938, **32**, 1252; Gillam, *Biochem. J.*, 1938, **32**, 1496 ; Lederer, Verrier and Hüttrer, *Bull. soc. Chim. biol.*, 1939, **21**, 629). Edisbury *et al.* showed that the non-saponifiable extracts from goldfish eyes exhibited maxima at 350mμ and 288mμ. Under the action of alcoholic hydrogen chloride, cyclisation occurred and narrow absorption bands appeared with maxima at 391, 369, 349 and 334mμ. As these bands also occur when vitamin A is treated in the same way, the structural difference between vitamins A and A_2 can only be small. In many species both vitamins occur, but it is noteworthy that the ratio, vitamin A_2/A, tends to be much higher in the liver than in the intestines.

It has not yet been possible to isolate pure vitamin A_2, but parallel experiments on concentrates rich in A and A_2, respectively, have been carried out by Heilbron and his colleagues. Ozonisation of vitamin A_2 yields geronic acid, indicating the presence of a β-ionone ring. A ketone $C_{25}H_{34}O$ is obtained by condensation of acetone with the aldehyde derived from vitamin A_2, and both the ultra-violet absorption and the degree of unsaturation of vitamin A_2 are consistent with a structure containing six conjugated double bonds ($C_{22}H_{31}OH$) :



. vitamin A_2 (?)

This formula aroused some doubts (Euler, Karrer and Solmssen, *Helv. Chim. Acta*, 1938, **21**, 211), but it is now clear that the ultra-violet spectrum falls into line with the spectra of the β-*apo*-carotinals prepared from β-carotene by oxidation (Karrer, Ruegger and Geiger, *ibid.*, 1171). The fact that little separation of A and A_2 occurs in molecular distillation is not, however, quite consistent with the above formula, and the precise structure is an open question.

Karrer, Geiger and Bretscher (*ibid*, 1941, **24**, 161 E) propose the formula

$Me_2C:CH.CH_2.CH_2.CMe:CH.CH:CH.CMe:CH.CH:CH.CMe:CH.CH_2OH$

and state that although A_2 may be important for fishes it is not active in mammals.

Lederer and Rathmann (*loc. cit.*) called attention to the fact that the ratio $E^{1\%}_{1cm.}$ 693mμ / $E^{1\%}_{1cm.}$ 620mμ in the colour test is not an accurate measure of the ratio, vitamin A_2/vitamin A. Just as vitamin A gives rise to two bands, one at 620mμ and the other (partially masked and showing as an inflexion) at 583mμ, so also vitamin A_2 shows a band at 645mμ in addition to the main 693mμ maximum. The absorption spectrum of the greenish-blue coloured solution ($SbCl_3$–vitamin A_2) extends to 620mμ, and in the absence of vitamin A the ratio $E^{1\%}_{1cm.}$ 693/620mμ is probably *ca* 7 : 1.

The ratio, **concentration A_2/concentration A**, will then be given by

$$\frac{E_{693m\mu}}{E_{620m\mu} - \frac{1}{7} E_{693m\mu}}$$

if it can be assumed that $E^{1\%}_{1cm.}$ 693mμ for pure vitamin A_2 is the same as $E^{1\%}_{1cm.}$ 620mμ for vitamin A. This assumption has much to recommend it. Lederer and Verrier (*loc. cit.*) find that the natural colour-test inhibitors contained in recovered fatty acids, cause the intensity of the 693mμ band to diminish greatly without affecting the 645mμ absorption to any appreciable extent. The latter band then stands revealed and may be displaced to 635mμ or 655mμ, depending on the proportions of inhibitors occurring in unsaponified oils. Oxidation of oils also diminishes the intensity of the 693mμ band rather than that of the 645mμ band, but thorough saponification causes the former to appear at full intensity in non-saponifiable fractions. In an oil from pike perch (*Sander lucioperca*) the following data were obtained:

	$E^{1\%}_{1cm}$ 695mμ/650mμ	695mμ/620mμ	bands visible at
before saponification	1·00	0·58	650, 610mμ
after ,,	1·8	2·63	695mμ

It is therefore essential to saponify the oils thoroughly and to use the nonsaponifiable fraction if the colour test is to be made quantitative.

Vitamin A_2 normally occurs only in fish, and predominates over vitamin A only in fresh-water fish. Mammals seem to be able to use it in place of vitamin A and to accumulate it, at any rate temporarily, in the liver. Frogs and rats under experimental conditions may use and store vitamin A_2, but only when the vitamin is added to the diet.

The proportions of vitamin A and A_2 present in the livers of fresh-water fish are highly variable; the ratio E693mμ/620mμ is highest, namely between 2 and 3 in carnivorous fishes (pike, *Esox lucius*; pike perch, *Sander lucioperca*; perch, *Perca fluviatilis*; and the wels, *Silurus glanis*). Omnivorous fishes (bream, *Abramis brama*; carp, *Cyprinus carpio*; tench, *Tinca tinca*, etc.) and the migratory fishes (salmon, *Salmo salar*; sturgeon, *Acipenser sturio*; eel, *Anguilla vulgaris*; trout, *Salmo fario*, and *Salmo irideus*) contain both vitamins, but the A_2 content is relatively smaller. Within a given species of fresh-water fish, the A_2/A ratio is fairly constant and is practically independent of the sex and age of the fish and of the seasons, and varies little with country of origin. The ratio E693/620mμ is normally highest in extracts from the liver and pyloric caeca or other absorptive parts of the gut, but Lederer records that carp show a ratio of 2·3–2·5 in the retina as against 0·5–0·9 in the

liver, so that the porphyropsin cycle may obtain for fishes with relatively low liver stores of vitamin A_2.

TABLE VI

After Lederer and Rathmann

Vitamin A_2 in pike perch (*Sander lucioperca*)

	$E_{693m\mu/620m\mu}$	p.p.m. vitamin A_2 fresh organs	mg. per fish
Liver	2·4	60	0·4
Stomach	1·0	1·4	0·01
Pyloric caeca	2·25	165	1·1
Pericaecal fat	1·0	6	0·09
Caecum wall	2·4	125	
Caecum contents	2·1	34	

In herbivorous animals it seems certain that vitamin A accumulates in the liver as the result of fission of provitamins such as β-carotene (the site of the process being uncertain) and it is probable that this can occur in most mammals. Because of the very large reserves of vitamin A which occur in many fishes (*e.g.* halibut, see p. 62), very considerable quantities of provitamins might be expected to occur in their food. Many workers (Drummond and Hilditch, Empire Marketing Board Rep., No. 35; Edisbury *et al.*, *loc. cit.*, and Lederer, *Bull. soc. Chim. biol.*, 1938, **20**, 554, 567) have found surprisingly low concentrations of the known provitamins in phytoplankton and zooplankton, and it seems possible that the poly-ene pigments of the astaxanthin type, which are so plentiful in plankton, may be utilised by marine fishes.

The origin of vitamin A_2 in fresh-water fishes is equally obscure. On one view of its structure, it could arise by an asymmetrical hydrolytic fission of β-carotene or similar provitamins:

$$C_{40}H_{56} \xrightarrow{2H_2O} C_{22}H_{32}OH + C_{18}H_{26}OH \quad \text{(hypothetical equation)}$$

If the provitamin is symmetrical, as in β-carotene, one might expect to obtain two absorbing substances, one an unsaturated alcohol with six conjugated double bonds and the other with four. The smaller molecule might be a lower homologue of vitamin A, in which case $\epsilon_{max.}$ would be perhaps half that shown by vitamin A_2. The actual spectrum shown by a vitamin A_2 concentrate frequently shows two maxima, that at 350mμ

being much stronger than that at 286mμ. The material absorbing at 284-285mμ is probably not a simple homologue of vitamin A, but from its distribution may well be a related substance. It has recently been shown (Morton and Creed, *Biochem. J.*, 1939, **33**, 318) that perch and dace kept in captivity and fed on a diet of blowfly larvae, appear to thrive for a considerable time. When this diet is enriched with carotene for a few weeks the storage of both vitamins A and A_2 increases considerably. Carotene can thus act as provitamin A_2 as well as provitamin A. This experiment does not necessarily imply that carotene is the main natural provitamin responsible for the liver reserves of fishes, but it does, however, indicate that the structures of vitamin A and A_2 must have a good deal in common.

The pigment of lobster (*Astacus gammarus* L.) is of special interest because it is probably the commonest carotenoid in the sea. Kuhn and Lederer (*Ber.*, 1933, **66**, 488) obtained a deep-green colloidal solution by grinding lobster eggs with sharp sand and water. The solution shows an absorption band with λ_{max} 500mμ and end-absorption in the red part of the spectrum. By adding ammonium sulphate to saturation, the green pigment is thrown out of solution and may be redissolved in water. Kuhn and Sörensen (*Angew. Chem.*, 1938, **51**, 465) find that this pigment—*ovoverdin*—may be purified by a procedure which they describe as being applicable to many chromoproteids, enzymes and other protein complexes. The pigment is adsorbed on aluminium hydroxide and eluted by means of solutions of ammonium sulphate less concentrated than those which effect precipitation. Ovoverdin may be eluted with 40%-saturated ammonium sulphate and reprecipitated by raising the salt concentration to 65% of saturation. The precipitate is dissolved in water, again adsorbed on alumina, eluted and reprecipitated. Rapid and effective fractionation of proteins can be carried out in this way. (Thus Birköfer is stated by Kuhn and Sorensen to have obtained the yellow enzyme from yeast, in a state of purity, using this method only. The enzyme preparation was obtained in good yield and the molecular weight of 70,000 agreed with earlier estimates.) Ovoverdin is found to contain carotenoid pigment and protein in the ratio 1 : 242, or 1 molecule of carotenoid to 2 of protein of molecular weight 72,000.

Preparations of ovoverdin are split up into a reddish pigment and protein by treatment with alcohol or acetone or by gentle heating. Acids and alkalies also cause a colour change from green to red with precipitation of protein. Acetic acid effects the colour change without throwing the protein out of solution. The dark-brown or bluish armour of the lobster also seems to contain a very similar chromoproteid, but extraction is

difficult because of the surrounding deposit of calcium salts. Treatment with dilute hydrochloric acid changes the colour to red and the carotenoid pigment may then be extracted by means of acetone or other organic solvents. The acetone extract is orange-yellow in colour, and after dilution with water all the pigment may be extracted by means of petrol ether. The petrol retains nearly all the colour on partition with 90% methyl alcohol. On treatment with alcoholic caustic soda, the resultant pigment can be separated into two fractions by partition between petrol and 90% methyl alcohol:

(i) petrol layer: 1% of the colour, $\lambda\lambda_{max.}$ 483, 448mμ (carotene).

(ii) alcoholic layer: 1 broad absorption band 350–450mμ; on acidification (acetic acid), small crystals appear which may be purified by recrystallisation from pyridine. The product is known as astacene and a few mgs. can be obtained from a lobster.

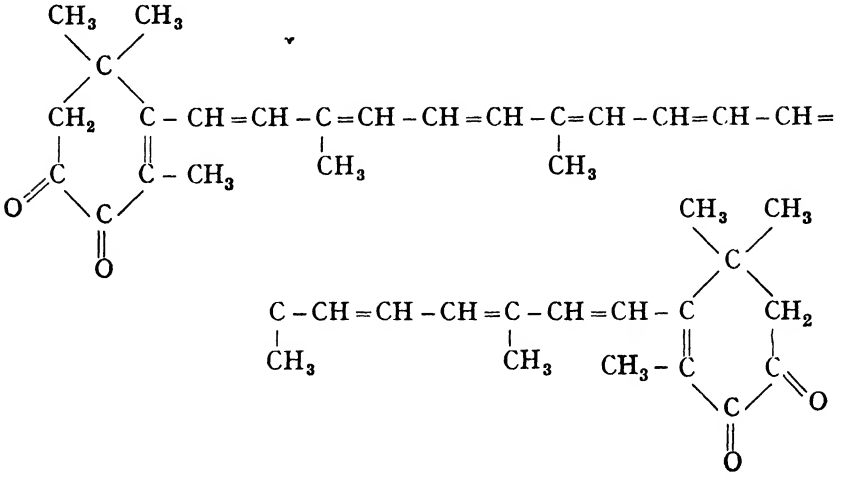
The red pigment obtained from ovoverdin, by direct extraction of the green eggs with acetone, may be extracted from the diluted acetone by means of petrol, but on shaking the petrol extract with 90% alcohol, nearly all the pigment is transferred to the alcohol layer. On addition of water, the pigment is precipitated as tiny sparkling bluish-violet crystals, which may be recrystallised from aqueous pyridine. This "ovo-ester" (Kuhn and Lederer, *loc. cit.*) yields astacene on treatment with alkali.

armour	hypodermis	eggs
dark-brown chromoproteid	red lipochrome (insoluble in water)	green chromoproteid (soluble in water)
HCl | acetone ↓	↓ extracted with acetone	↓ acetone
red epiphasic carotenoid	red epiphasic carotenoid	red hypophasic ovo-ester
↓ NaOH	↓ NaOH	↓ NaOH
astacene	astacene	astacene

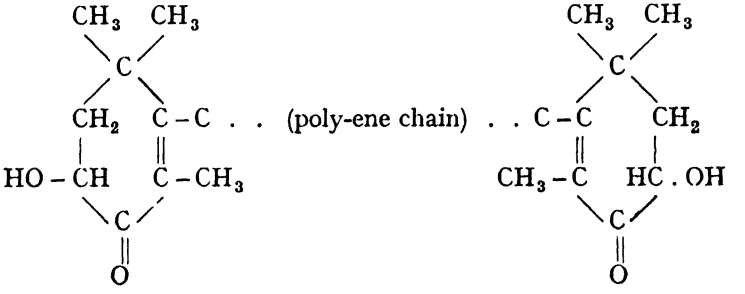
Astacene shows a broad unresolved absorption band, $\lambda_{max.}$ 500mμ, in pyridine.

The so-called ovo-ester is not actually an ester but a free xanthophyll, $C_{40}H_{52}O_4$, designated astaxanthin by Kuhn and Sörensen (*loc. cit.*). It differs from astacene, $C_{40}H_{48}O_4$, by 4 hydrogen atoms. The action of alkali is not a saponification but an autoxidation of a double α-ketol, since the transformation to astacene requires 2 mols of oxygen and results in

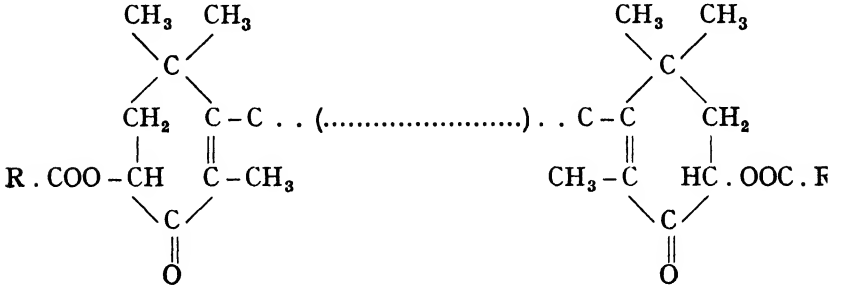
2 mols. of hydrogen peroxide. Karrer (*Helv. chim. Acta*, 1934, **17**, 745) has shown that astacene is a tetra-keto β-carotene:



so that astaxanthin will be



and its esters



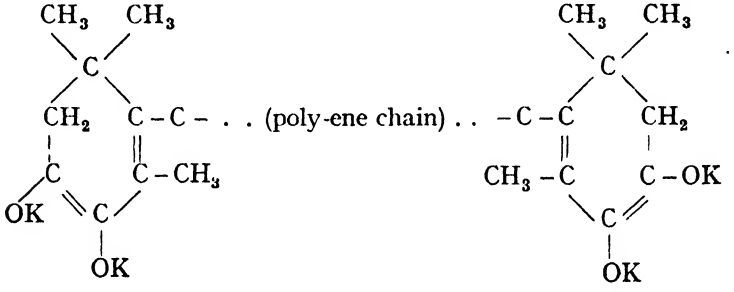
R = CH_3, hypophasic ester.

R = $C_{15}H_{31}$, epiphasic ester.

It is probable, but not quite certain, that the carbonyl group rather than the hydroxyl group in astaxanthin is conjugated to the poly-ene chain. The autoxidation of astaxanthin in alkaline solution resembles that of benzoin to benzil:

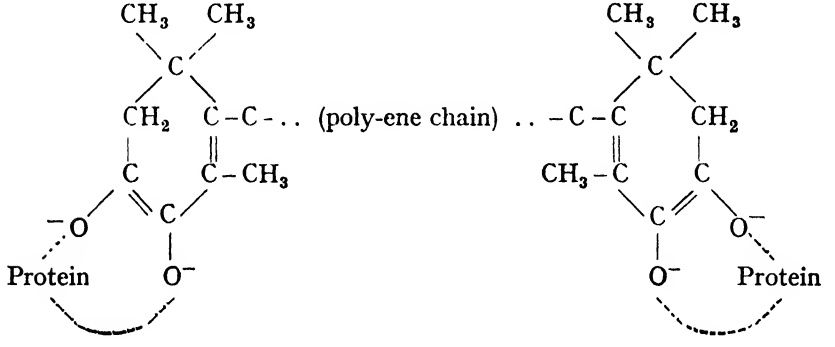
$$\mathrm{Ph.CHOH.CO.Ph} \longrightarrow \mathrm{Ph.C(OK)=C(OK).Ph.} \longrightarrow \mathrm{Ph.CO.CO.Ph.}$$

In the absence of air, astaxanthin gives a deep-blue potassium salt:



and on entry of air the colour changes to red with formation of astacene.

Ovoverdin will, on this basis, be a derivative of the enolic form of astaxanthin:



There may be two protein molecules of mol. wt. *ca* 70,000, or both ends of the carotenoid may be linked to one protein molecule of mol. wt. *ca* 140,000. The assumption of an ionic linkage between protein and carotenoid is necessary if the colour change which occurs on fission of the complex is to be explained. Kuhn and Sörensen point out that the reaction between riboflavin and its specific protein at p*H*7 effects changes in absorption spectrum and fluorescence similar to those caused by alkali at p*H*11. The hypothesis of a salt-like link between the protein and the

prosthetic group does not, however, explain the immunity of the blue-green chromoproteid to autoxidation.

The red epiphasic pigments of the lobster hypodermis are esters of astaxanthin and not of astacene as had been thought hitherto. On saponification in the absence of air, they give the deep-blue enol salt which is easily oxidisable to the tetraketone. It is likely that the pigments of crustaceans generally are astaxanthin derivatives and that astacene is essentially an artefact. Wald (*Nature*, 1937, **140**, 197) has isolated astacene from the retinas of birds, and it appears now that astaxanthin-protein complexes may be concerned in visual processes.

This work has been described in some detail because it clarifies a difficult field of research which is of undoubted importance. The carotenoids of zooplankton may possibly provide the provitamin A responsible for the immense reserves of fishes.

Because of the risk of autolysis, the freshly collected minute crustaceans, etc., have hitherto been preserved in alcohol, at the price of considerable denaturation. The new technique worked out for ovoverdin reduces the risk of detaching prosthetic groups from conjugated proteins and opens up new possibilities. Astaxanthin is not, from its structure, likely to be a provitamin A, but Wald's isolation of astacene from retinas is a hint that fresh surprises may be met with in this field.

Analytical Problems

The analysis of materials for vitamin A activity has proved a formidable problem. Many difficulties have been encountered in developing a more quantitative technique for bio-assays than the earlier rather approximate one. Much attention has been given to basal diets, variability of response and statistical interpretation of data (see Medical Research Council Special Report, Series No. 202). The effort to substitute physical and chemical tests has had a good deal of success, but the biological assay is by no means obsolete, although it will continue to need time and skill and cannot but be costly. Unfortunately, the biological test does not discriminate between vitamin A and provitamins A. Furthermore, recent research has emphasised a complication of very great importance, namely a wide variability in the utilisation of β-carotene (and other provitamins A), depending upon the form in which it is administered. When the chemist analyses a given product for vitamin A or provitamin A he aims at a precise determination of the actual amounts present, whereas the biochemist's animal assay is concerned with *available* vitamin A or carotene. If a large proportion of the total is nutritionally useless, as often happens, it may be necessary to change the method of preparing or cooking the food, or to alter the bulk composition of the diet so as to

improve utilisation. In short, the problem of vitamin A nutrition has emerged from a phase of spurious clarity, resulting from over-simplification, into a very complex phase in which the main task is the two-fold one of refining both analytical methods and biological methods without confusing the many variables or failing to stress the distinction between vitamins and provitamins A.

Any form of assay must be considered in relation to the enormous range over which vitamin A concentration is spread:

Milk	about 1	I.U./g.	
Butter	10–50	,,	
Cod liver oil	300–3000	,,	
Halibut liver oil	10,000–150,000	,,	
Special liver and fish intestinal oils	up to 500,000	,,	
Rich concentrates	,, 3,300,000	,,	(approx.)

In vegetable products the potency is due to carotenoid provitamins, and it is necessary to ascertain (*a*) the gross provitamin content and (*b*) the availability of the provitamin (this will depend on the physical condition of the active agent and upon several other factors). Here, the importance of refined chemical methods is obvious, not only from the point of view of knowing exactly what is administered to the animal but also from the aspect of analysing blood, liver, faeces and urine in order to follow the fate of the provitamin. It is necessary to rely upon colorimetric or spectrophotometric determinations carried out upon extracts which have been freed from biologically inert " xanthophylls ". Even then, it is desirable in accurate work to ascertain the proportions of α- and β-carotene since the former is only half as potent as the latter, and sometimes it is also necessary to estimate kryptoxanthin. The quantitative separation of related carotenoids demands the use of chromatographic analysis and it is not always easy to avoid the production of artefacts which falsify the results. In butter and milk, the problem is to estimate *both* vitamin A and carotene.

The whole question of vitamin A determination was for long hampered by the failure to obtain vitamin A or its esters in crystalline form. This meant that there was no fixed standard of reference. On the other hand, carotene was available as an apparently pure substance possessing vitamin A activity. Accordingly the 1931 Conference on Biological Standards (League of Nations Health Organisation) adopted " carotene " as the Standard and defined one International Unit as the activity of 1 microgram (1μg.) of the standard preparation of " carotene ". By 1934, it had become clear that " carotene " as previously known, was a mixture

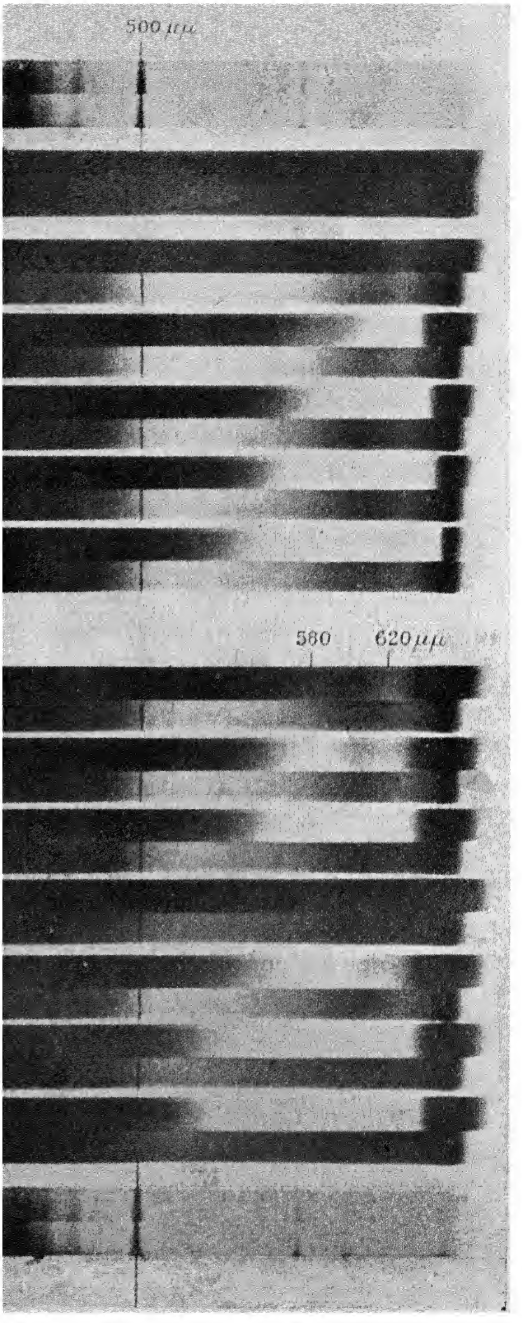
of α- and β-isomerides differing in potency. It appeared that 0·6μg. of pure β-carotene could be equated with 1μg. of the 1931 standard preparation. The International Unit was then re-defined as the vitamin A activity of 0·6μg. of pure β-carotene, the potency of which was thus $1{\cdot}66 \times 10^6$I.U./g. Implicit in such a standard is the idea of a substantially constant efficiency of assimilation and conversion to vitamin A of β-carotene fed, just at the onset of a decline in weight and at the correct dosage, to rats, on a suitable basal diet. Stoichiometric conversion may not occur, but constancy of conversion is a precondition of success as a Standard. When pure crystalline vitamin A became available it would be possible (according to the line of thought followed in 1934) to carry out biological tests using β-carotene and vitamin A side by side. In this way the potency of vitamin A in I.U./g. could be ascertained. From that point onwards there would be no logical difficulty in the way of using physical or chemical methods for determining the percentage of β-carotene or vitamin A and then expressing the results in terms of I.U./g.

The available physical and chemical methods depended upon either ultra-violet absorption spectra or the antimony trichloride colour test; and in the absence of well-defined criteria of purity for vitamin A, it was clearly desirable to link the spectrophotometric data and colour-test data directly with vitamin A potencies, determined by feeding trials in which liver oils and β-carotene were compared side by side. The potency of pure vitamin A, when suitable preparations became available, could then be obtained either by extrapolation from physical data or by fresh bio-assays against β-carotene.

At this point it is desirable to describe the physico-chemical tests in detail before completing the discussion on standardisation. The vitamin A absorption band, with λ_{max} 325mμ, and the antimony trichloride blue colour, both vary in intensity with vitamin A concentration, but both tests are subject to certain limitations:

(i) The absorption spectrum of a liver oil is made up of a summation of (*a*) the intrinsic absorption of vitamin A and its esters and (*b*) irrelevant absorption due to unsaturated glycerides, anti-oxidants and other substances. Uncertainty concerning the magnitude of the irrelevant absorption means that $E^{1\%}_{1cm.}$ 325mμ measured on the oil merely indicates an upper limit to the possible vitamin A concentration.

(ii) The colour test on oils is carried out as follows: a suitable solution of oil in pure chloroform (*e.g.* 20% wt./vol. of cod liver oil or 0·1–0·2% wt./vol. of halibut liver oil) is prepared, and a known



The absorption spectrum of a blue solution obtained by treating a vitamin A concentrate with the antimony trichloride reagent. The photograph shows $\lambda_{max.}$ 620 mμ with an inflexion near 583 mμ.

In these photographs the 620 mμ band has been greatly inhibited, and the suppression of the masking effect of the main band permits the selective absorption at 583 mμ to appear as a fully developed band.

(*Biochemical Journal*, 1931, **25**, 1360)

PLATE VI

volume (0·2 or 0·4 c.c.) is mixed with 10 times its volume of the Carr-Price $SbCl_3$ reagent. (It is often desirable to add 1 or 2 drops of acetic anhydride.) The mixture is at once poured into a cell already in position in a colorimeter or spectro-photometer. Using cod liver oil, the resulting blue solution shows an absorption band with a maximum near 605mμ and an inflexion near 572mμ. The intensity of absorption is measured within 15 secs. of mixing, because the colour is transient.

Example:

20% wt./vol. cod liver oil in $CHCl_3$ (solution A)

0·4 c.c. (A) + 4 c.c. $SbCl_3$ reagent, 2 cm. cell

E at 605mμ 2·1, at 572mμ 1·7

concentration in cell: $20 \times 0{\cdot}4/4{\cdot}4 = \frac{20}{11}\%$

$$E^{1\%}_{1\text{cm.}} = \frac{2{\cdot}1 \cdot 11}{2 \times 20} = 0{\cdot}58 \text{ at } 605\text{m}\mu$$

$$= \frac{1{\cdot}7 \times 11}{2 \times 20} = 0{\cdot}47 \text{ at } 572\text{m}\mu$$

i.e. $E^{1\%}_{1\text{cm}}$ 605mμ 0·58; 572mμ 0·47

As Wolff and his colleagues pointed out, the colour test is seriously affected by the presence of natural inhibitors, the rôle of which can be artificially simulated by the addition of methyl-indoles in small quantity (Eekelen, Emmerie, Julius and Wolff, *Acta Brev. Neerland.*, 1931, **1**, 8; Morton, *Biochem. J.*, 1932, **26**, 1197). The natural inhibitors are present in particularly high concentration in cod liver oils prepared by cooking fresh livers in sealed tins (Mittelmann, *Trans. Inst. Explor. of the North*, Leningrad, 1927, **38**, 39; Lovern, Creed and Morton, *Biochem. J.*, 1931, **25**, 1341). On opening a tin, the liver oil is easily separated and gives an unusually feeble colour test. On standing, the oils gradually acquire the power to react normally towards the $SbCl_3$ reagent, as the concentration of inhibitor spontaneously diminishes. The practical consequence is that quantitative determination of vitamin A in low potency oils calls for modified procedures because both ultra-violet absorption and the colour test are subject to variable interference. The remedy lies in careful extraction of the non-saponifiable fraction, whereby the irrelevant ultra-violet absorption and the colour-test inhibitors are both eliminated.

Saponification: 0·2–1·0g. of oil (according to the potency indicated by a rough colour test) is washed into a flask (100 c.c. R.B) with alcohol (100 c.c.) and 10N KOH(0·5 c.c.) and the mixture boiled on the water bath with shaking until clear (5 minutes). The solution is poured into a separating funnel and the flask is washed out with 20 c.c. water followed by 25 c.c. freshly re-distilled, peroxide-free ether. The funnel is gently agitated and allowed to stand for 3 minutes. The

ether layer is collected and the soaps re-extracted with a second (and if necessary a third) portion of 25 c.c. re-distilled ether.

The combined ethereal extract is gently washed with distilled water, followed by N/2 aqueous KOH, and again twice with 15 c.c. portions of distilled water. The washed extract is run into a flask and the funnel washed with a mixture of alcohol and ether The greater part of the ether is distilled off and the remainder removed in a current of N_2 or CO_2 (Dehydration of vitamin A concentrates is most readily accomplished by adding a little absolute alcohol and " blowing " on a water bath by evaporating in a stream of N_2 from a cylinder. By repeating the operation two or three times using quite small quantities of alcohol, traces of moisture or ether soon disappear ; porous tile should not be used.) The residue should be dissolved in absolute alcohol and made up to a known volume (*e.g.* 25 c.c). An aliquot portion (*e.g.* 5 c.c.) is retained and diluted so that $\log I_0/I$ at 325mμ falls near 1·2 for the cell thickness in use The remaining aliquot is again sent down to dryness in a current of nitrogen and the residue taken up in pure chloroform ready for the colour test. (Pure hexane or cyclohexane, specially purified for spectroscopy, is often used instead of alcohol as the solvent for determining ultra-violet absorption.)

The following figures (which were obtained on the Reference Cod Liver Oil used by the United States Pharmacopoeia as a standard, secondary to β-carotene, see p. 85) illustrate the value of saponification :

Tests carried out on the oil :

$$E^{1\%}_{1cm}\ \begin{matrix} 606m\mu \\ 572 \end{matrix}\Big\}\ \text{colour test}\ \begin{cases} 3\cdot1 \\ 2\cdot1 \end{cases}$$

328mμ ultra-violet test 1·74

Test, carried out on the non-saponifiable fraction :

		1934	1937
$E^{1\%}_{1cm}$ 620mμ / 583mμ	colour test	4·85 / 2·35	4·4 / 2·28
328mμ	ultra-violet test	1·58	1·39

A more typical cod liver oil might give $E^{1\%}_{1cm}$ 328mμ 0·9 on the oil and 0·7 on the non-saponifiable fraction. Judged by the data on pure vitamin A (p. 95), the non-saponifiable fraction yields reasonably good results because

(*a*) the colour test maximum occurs in the right place, *i.e.* 615–620mμ as against 600-610mμ.

(*b*) the colour test, which is inhibited when unsaponified oil is used, now shows increased absorption and the ultra-violet test shows diminished absorption, so that the two tests agree :

	pure vitamin A	U.S.P. oil	% vitamin A in U.S.P. oil	
$E^{1\%}_{1cm}$ 620mμ	6000	4·85	$\frac{4\cdot85}{60}=0\cdot81$	*i.e.* 0·825% in 1934
$E^{1\%}_{1cm}$ 325mμ	1880	1·58	$\frac{1\cdot58}{18\cdot8}=0\cdot84$	
		4·4	$=0\cdot733$	0·735% in 1937
		1·39	$=0\cdot74$	

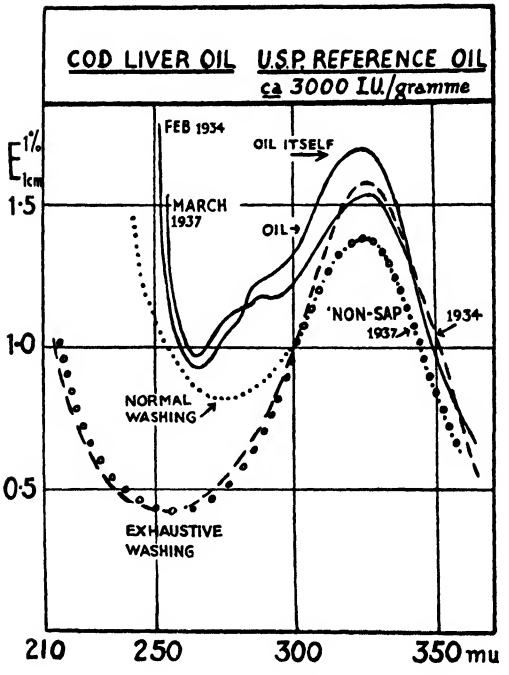


FIG. 37

After Morton, vE Congrès International Tech. et Chim. Ind. Agricoles Scheveningue, 1937, *Comptes rendus*, vol. I, p. 58

These curves illustrate the spectrophotometric determination of vitamin A. The international unit of vitamin A activity is that of 0·6γ of pure β-carotene The United States Pharmacopoeia Reference Oil is a subsidiary standard, which possessed when first issued 3,000 I U. per gram determined by biological assays using the carotene standard of comparison.

The upper curve (Feb 1934) represents the spectrum of the oil, and the - - - curve that of the non-saponifiable fraction It will be seen that the peak is lowered a little and the short wave absorption very considerably. The difference indicates the elimination of irrelevant absorption.

The lower continuous curve shows how the Reference Oil had deteriorated a little by 1937. The —o—o— curve again demonstrates the removal of irrelevant absorption, whilst the comparison of this curve with the dotted portion illustrates how the persistence ($E_{max.}$ minus $E_{min.}$) depends on thorough washing of the ethereal solution of the " non-sap." " Pure " vitamin A exhibits $E^{1\%}_{1cm.}$ of the order 1,600, so that $E^{1\%}_{1cm.}$ 328mμ 1·6 means that the Reference Oil contained *ca* 0·1% vitamin A.

In the period between 1931 and 1934 many fish liver oils have been subjected to bio-assay against standard carotene, and there was a considerable body of evidence to show that an absorption density of 1 at 325mμ could be equated to 1,600 I.U./g.

The 1934 Conference recommended "that a sample of cod liver oil, the potency of which has been accurately determined in terms of the International Standard Preparation of β-carotene, shall be provided as a Subsidiary Standard of Reference". The Report went on: "in view of the fact that the Reference Cod Liver Oil of the United States Pharmacopoeia, which has been accurately assayed in terms of the provisional International Standard adopted in 1931, has been in effective use in the United States of America for some time, the Conference recommends that the Board of Trustees of the United States Pharmacopoeia be approached and invited to place a quantity of their Reference Cod Liver Oil at the disposal of the Health Organisation of the League of Nations, with a view to its adoption for international use as a Subsidiary Standard for Vitamin A."

The Conference also decided:

"It has been found that, within certain defined conditions, measurement of the coefficient of absorption E at 328mμ affords a reliable method for measuring the Vitamin A content of liver oils and concentrates. As a means of converting values obtained for $E^{1\%}_{1cm.}$ 328mμ into a figure representing the International Units of Vitamin A per gramme of the material examined, the factor 1,600 is recommended for adoption." In a note on the technical procedure for the spectrophotometric determinations, it was pointed out, firstly, that the intensity of absorption at 328mμ may be determined to within ±2·5% by any of the recognised methods of spectrophotometry; and, secondly, "the factor 1,600 is the average figure derived from a series of comparable and independent tests on the unsaponifiable fractions of liver oils and on concentrates of high potency. It is desirable that, when a figure expressing the biological potency of a preparation has been derived by the use of this calculation, the fact should be stated."

Subsequent events are worth discussing in some detail because many important issues in spectrophotometry have been raised.

In point of ease and convenience the spectrophotometric method has obvious advantages, but much will depend upon the accuracy of the conversion factor. The early work (M.R.C. Rep. 202, already cited) was based largely on the 1931 standard carotene, and experience with β-carotene standard had still to be gained. The first account of co-operative work appeared in 1937 (E. M. Hume, *Nature*, **139**, 46). The Vitamin A Sub-Committee appointed by the Medical Research Council and the Lister Institute, organised co-operative biological assays (9 laboratories) with spectrophotometric controls of the materials under test. A special dilution of the β-carotene standard was used and its stability was checked spectrophotometrically at the beginning and the end of the experiment. A specimen of halibut liver oil was obtained and a concentrate (non-saponifiable fraction) was prepared from it. Both materials were diluted for test in a central laboratory and tested at intervals by means of ultra-violet absorption at 325mμ so that any deterioration could be recognised. The biological activity of the concentrate was found to be lower than that of the oil, but the spectroscopic tests indicated satisfactory stability for the oil and considerable deterioration for the concentrate during the test period. The tests were completed in October 1936 and led to a provisional conversion factor of 1,470 (range of individual values 1,400–1,700), later corrected to 1,570 as a result of statistical analysis by J. O. Irwin

(Hume, *Nature*, 1939, **143**, 22). This test, it must be emphasised, was based on a direct comparison between the halibut liver oil and the β-carotene standard preparation. The fact that the conversion factor agrees so closely with 1,600 is no doubt partly accidental, because the " equality " of 0·6μg. β-carotene and 1μg. of the 1931 standard carotene was something of an approximation.

It is necessary now to turn to the history of the United States Pharmacopoeia Reference cod liver oil (for brevity U.S.P. Oil I). The biological potency assigned to the oil at the time it was issued was 3,000 I.U. per gm., based on the average of results collected from nine different laboratories by E. M. Nelson (tests against 1931 standard carotene). In 1934 the absorption at 328mμ shown by the non-saponifiable fraction of U.S.P. Oil I was $E^{1\%}_{1cm.}$ 1·58 and a conversion factor 3,000/1·58 or 1,900 was indicated. A conversion factor 2,000 has in fact been widely used in America.

It was clearly desirable to subject the Reference Oil to assays against the 1934 β-carotene standard preparation. Ten laboratories participated in co-operative tests which were reported upon by Miss Hume in 1939 (*Nature*, **143**, p. 22). The potency of the oil appears to have fallen since it was first issued :

Dr. Nelson's series	Miss Hume's series
2,400–3,725 I.U./g. (average 3,000)	1,334–3,270 I.U./g. 6 out of 10 values : 2,200–2,600 I.U./g. weighted mean, 2,619 I.U./g. ; mean limits of error per experiment (P = 0·99) 73 – 137%

The intensity of absorption (determined on the non-saponifiable fraction) had decreased during the period in between the two sets of experiments from 1·58 to 1·4 on the non-saponifiable fraction. A conversion factor of 1870 for the U.S.P. Oil I was therefore indicated by the co-operative tests against β-carotene.

In addition to British co-operative tests, there has been much activity in the United States of America. Barthen *et al.* (*J. Amer. Pharm. Assoc.*, 1937, **26**, 515 ; 1939, **28**, 661) carried out collaborative tests on the spectrophotometric method. Accepting without qualification 3,000 I.U./g. as the potency of the U.S.P. Oil I, this group of workers concerned itself with the diversity of instruments (and results) in spectrophotometry. Each laboratory was advised to determine the E value of the U.S.P. Oil I, and then to divide into 3,000 so as to obtain a conversion factor for the instrument in use.

"To achieve the greatest degree of accuracy in results, the E value of the U.S.P. reference oil, its unsaponifiable fraction and/or any other suitable standard should be determined every day and the conversion factor calculated to check the constancy of conditions necessary for accurate operation of the instrument. In this manner, errors due to conditions in the laboratory, the instrument or the human element can at least be reduced to a minimum. However, it is obvious that expertness, as a consequence of experience in the technique and knowledge of the fundamental principles of the instruments, is an essential factor for the attainment of accurate results Under specified and well-controlled conditions of operation of the physical instruments, capable of accuracy of measurement, results can and should be obtained which are in closer agreement than those obtainable by biological assay."

In this work the U.S.P. Oil I was examined as oil (not as non-saponifiable fraction) in 7 laboratories using spectrophotometers :

$$\text{mean } E^{1\%}_{1\text{cm}} \ 328\text{m}\mu, \ 1{\cdot}545$$

and a 50% dilution in cottonseed oil gave a mean value $E^{1\%}_{1\text{cm.}}$ 0·866. Now it is obvious that the cottonseed oil solution absorbs more than half as strongly as the U.S.P. Oil I, so that the diluent must contribute irrelevant absorption. This irrelevant absorption vitiates the direct comparison with biological assays. In addition, the oil itself possesses irrelevant absorption which is eliminated when the test is carried out on the non-saponifiable fraction.

Comparing this work with that of Holmes *et al.* (*J. Amer. Pharm. Assoc.*, 1937, **26**, 523), in which all laboratories tested altogether 34 samples, it was considered that "In order to correlate the data of different laboratories it is essential to refer the data of all laboratories to a standard of reference, the U.S.P. Reference Cod Liver Oil being well suited ".

The average E value was, however, 1·61 as against 1·545 (Barthen *et al.*, 1939). The oil had obviously deteriorated by 1939 but both sets of workers regarded it as possessing a potency of 3,000 I.U./g. Both groups were concerned with the gross absorption determined on the oil and made no assessment of irrelevant absorption.

McFarlan, Bates and Merrill (*ibid.*, 1940, **12**, 645), in another useful paper, called attention to the instability of the U.S.P. Oil I but found $E^{1\%}_{1\text{cm.}}$ 1·38–1·39 for the oil studied on the non-saponifiable fraction.

The available data on the U.S.P. Oil I may now be assembled :

U.S.P. Reference Oil I

Nominal Potency : (*a*) 3,000 I.U./g. (against 1931 Standard carotene),
(*b*) 2,619 I.U./g. (against Standard β-carotene). Vitamin A Sub-Committee 1934.

(*a*) and (*b*) are in reasonably good agreement considering the change in standard preparation and the time interval between the two sets of assays.

SPECTROPHOTOMETRIC DATA

	$E^{1\%}_{1cm.}$ 617mμ $SbCl_3$ colour test on non-sap	$E^{1\%}_{1cm.}$ 325mμ Spectrophotometric tests on oil	on non-sap	date	observers
1.	4·85	1·74	1·58	1934	Morton and Edisbury
2.	4·40	1·55	1·39	1937	ditto
3.		1·61		1936?	Holmes *et al.*
4.		1·545		1937	Barthen *et al.*
5.		1·59		1940	Ewing *et al.*
6.		1·62			
7.			1·346		
8.		1·555	1·38–1·39	1940	McFarlan *et al.*
9.		1·495	1·49*	1941	Demarest (*Ind. Eng. Chem., Anal. Ed.*, 1941, **13**, 374.)

* This may be an error.

A more recent paper (Ewing, Vandenbelt, Emmett and Bird, *Ind. Eng. Chem., Anal. Ed.*, 1940, **12**, 639), accepted the U.S.P. Reference Oil as 3,000 U.S.P. units/g. (by not identifying the U.S.P. unit with the I.U. the authors slip unobtrusively into safety, a small defect in a valuable paper). The intensity of absorption for the U.S.P. oil is given as :

$E^{1\%}_{1cm.}$ 325mμ { 1·59 (whole oil in isopropyl alcohol) / 1·62 (whole oil in ethyl alcohol) / 1·35 non-saponifiable fraction

From internal evidence, the measurements are almost certainly accurate to within ±2%. Six oils, carefully assayed by animal tests (assuming 3000 units/g. for the U.S.P. Oil I) gave a mean conversion factor of 2,152.

TABLE

Oil	Bio-assay U.S.P. units/g.	$E^{1\%}_{1cm}$ Spectrophotometer	Calculated Conversion Factor
U.S.P. Oil I - -	3,000*	1·35 (on non-sap.)	2222
Halibut - - -	65,000	31·46	2066
Mixed - - -	185,300	78·68	2355
Mixed - - -	295,100	128·60	2295
Halibut - - -	31,900	15·05	2120
Mixed - - -	87,800	39·85	2203
			mean 2152

* (Note 3000/2152 = 1·39.)

The spectroscopic data show beyond any doubt that deterioration has occurred. The frequent use of a conversion factor based on 3,000 I.U./g. involves two errors; it neglects irrelevant absorption, and it neglects deterioration. Thus $3000 \div 1{\cdot}6 = 1875$. There is, however, some compensation of errors. The use of E values on the non-sap. and 3,000 I.U./g. neglects deterioration and rests upon the assumption that 1μg. of the 1931 Standard preparation corresponds exactly with 0·6μg. of the 1934 β-carotene preparation. Thus $3{,}000/1{\cdot}39 = 2{,}152$. The use of E values on the non-sap. and 2,619 I.U./g. corrects for irrelevant absorption and makes use of the comparison with the 1934 Standard preparation:

e.g. $2619/1{\cdot}44 = 1820$ ($1{\cdot}44$ and $1{\cdot}39$ representing the range of
$2619/1{\cdot}39 = 1885$ E values at the time of the assays.)

It is interesting now to return to the paper of Barthen *et al.* The spectrophotometric data indicate that some of the participants were relatively new to the work, but the following selection from the results is illuminating:

TABLE

Material	Relative Potencies by bio-assays	$E^{1\%}_{1cm.}$ 325mμ mean	$E^{1\%}_{1cm.}$ 325mμ range	$E^{1\%}_{1cm.}$ 325mμ Calc.* from bio-assays	$E^{1\%}_{1cm.}$ 325mμ range permissible
U.S.P. Oil I	1	1·545	1·43–1·66	1·39	1·36–1·42
Halibut liver oil	23·33	30·31	28·35–33·3	32·43	31·73–33·13
Distillation product	100	141·98	134·1–152·8	139	136–142
† *Diluted materials*					
U.S.P. Oil I	0·5	0·866	0·83–0·909	0·695	0·68–0·71
Halibut liver oil	11·66	15·54	14·2–17·25	16·215	15·86–16·56
Distillation product	50	72·22	67·18–79·1	69·5	68–71

* Accepting 1·39 as the true value on the non-sap.
† Diluted with an equal volume of cottonseed oil.

It will be seen that a linear relationship between potency and E values requires the "*non-sap.*" E *value.* The conversion factor appropriate to this work is therefore

$x/_{1{\cdot}39}$ where x is the potency in I.U./g. of the U.S.P. Oil I,
thus if $x = 3000$ it is 2152,
$x = 2500$ it is 1800.

It will now be clear that the conversion factor for the effective use of the U.S.P. Oil I rests entirely, so far as published evidence goes, on E$1{\cdot}39 \pm 0{\cdot}03$ on the non-sap., and 2,619 for the potency against the β-carotene standard.

All the collaborative data published, both British and American, on this oil lead to 1,885 ±the inevitable error of bio-assays which is here as small as it can profitably be made.

Now the later tests undertaken by the Vitamin A sub-committee on vitamin A β-naphthoate have the advantage of being carried out on a nearly pure material, controlled spectrographically throughout the experiments.

For the three co-operative sets of assays the respective conversion factors are :

Halibut liver oil - - -	1,570
U.S.P. Oil I - - - -	1,820–1,885
Vitamin A β-naphthoate - -	1,600–1,700*

* Depending on whether **E** values are used on the ester freshly diluted in alcohol (or cyclohexane) *or* the ester as diluted for feeding tests.

Hence the final result of the British series is $1{,}690 \pm \begin{cases} 195 \\ 120 \end{cases}$

Data on the physical properties of pure vitamin A, based on the crystalline alcohol, the acetate, β-naphthoate and anthraquinone β-carboxylate, are accumulating and will no doubt be published in due course. From a careful consideration of those now available privately,

$$E^{1\%}_{1cm}\ 617m\mu\ 6{,}000 \pm 200 \text{ (colour test)}$$
$$325m\mu\ 1{,}880 \pm 40 \text{ (u.v. absorption)}$$

seem to be the best.

The potency of vitamin A is thus $1{,}690 \times 1{,}880 = 3{\cdot}18 \times 10^6$ I.U./g. ±*ca* 10%.

In effect, this means that the potency of vitamin A is practically indistinguishable from twice that of an equal weight of β-carotene. (In fact, if one molecule of carotene yields one molecule of vitamin A, the potency of vitamin A should be

$$\frac{1{\cdot}66 \times 10^6 \times 536}{286} = 3{\cdot}12 \times 10^6 \text{ I.U./g.}).$$

Summarising experience on the working of the recommendations of the 1934 Conference it may be said : (*a*) that British collaborative tests, laying emphasis on the β-carotene standard, indicate that the 1,600 conversion factor might profitably be raised to 1,700 ; (*b*) that American experience has very greatly increased the evidence in favour of an accurate linear relationship between **E** values and vitamin A potency.

The great variety of instruments which has arisen to meet the growing application of spectrophotometry to analysis has caused some confusion and a new respect for the difficulties in the way of high accuracy. At the

present stage of development, the views of many competent workers may be summarised as follows :

(1) Alignment of spectrophotometers should be checked daily, and with some instruments on every plate.

(2) The calibration requires the use of a solution, preferably in the same cell which is to be used for the vitamin solution under test.

(3) Vitamin A preparations, because of instability, are not wholly suitable for testing spectrophotometers.

(4) The solute used for calibration should be a pure substance, easily accessible and reasonably stable.

(5) The E value of the solute should be accurately known from concordant determinations made in different laboratories, using photo-electric spectrophotometric methods for standardisation.

(6) Potassium nitrate and potassium chromate are two such solutes which have proved satisfactory, especially the former.

(7) Neither potassium nitrate nor potassium chromate is very suitable for calibrating instruments of the type of the vitameter A.

The perfect calibrating solute will, then, be one which shows a well-defined absorption maximum at 325mμ, is easily accessible in a high state of purity and is reasonably stable.

Salicylaldehyde is such a substance: λ_{max} in alcohol 325mμ, logϵ *ca* 3·48. A second maximum occurs at 255mμ, log ϵ 4·0.

Anthraquinone seems to be a better choice. It possesses an excellent absorption band at 325mμ in alcohol and is probably easier to obtain and keep in a high state of purity than salicylaldehyde. Highly purified anthraquinone is just sufficiently soluble in hexane.

There are, however, no precise E values (*i.e.* photo-electric readings) on either substance, but the difficulty could be removed.

In the case of oils containing more than 10,000 I.U./g. it is rarely necessary to resort to extraction of the non-saponifiable fraction, because at 325mμ, irrelevant absorption is a negligible fraction of the observed value. If, however, the colour test appears anomalous, or does not agree with the ultra-violet assay, it is always safer to work on the non-saponifiable fraction. Serious difficulties are confined to oxidised oils or those subjected to drastic processing. Commercial whale liver oils are specially awkward because both the colour test and the ultra-violet absorption are usually anomalous (*cf.* Morgan, Edisbury and Morton, *Biochem. J.*,

1935, **29**, 1645; Pritchard, Wilkinson, Edisbury and Morton, *ibid.*, 1937, **31**, 258). Isolated instances have been reported in the literature of other mammalian liver fats showing absorption maxima at 290–300mμ instead of 325mμ. The non-saponifiable fractions show the same displaced absorption. In attempting to remove inert material from sterol-free mammalian concentrates, Pritchard *et al.* found that aqueous alcohol (83%) readily dissolved the main chromogenic constituent ($SbCl_3$ test), leaving behind about half the original material as a fraction showing $\lambda_{max.}$ 285–290mμ, and giving an abnormal colour reaction. Exhaustive extraction with 83% alcohol resulted in an apparently complete separation:

Fraction soluble in 83% alcohol. Behaves like a typical vitamin A concentrate		Fraction insoluble in 83% alcohol. No vitamin A bands (*i.e.* no maxima at 620 or 583mμ in the colour test, or at 325mμ)
$E^{1\%}_{1cm}$ 620mμ	1340	$\lambda_{max.}$ 290mμ $E^{1\%}_{1cm.}$ 240
583	740	$SbCl_3$ $\lambda_{max.}$ 594 and 496mμ
328	440	$E^{1\%}_{1cm}$ 180 172
		Biological test, 17,900 I.U./g.

The potency of the material insoluble in 83% alcohol is not due to contamination with vitamin A, and it appears probable that the new product possesses 1/10–1/20 the activity of vitamin A. Judging from the properties of vitamin concentrates prepared from the fresh livers of a number of mammalian species, the abnormal absorption at 290–300mμ instead of 325mμ is somewhat rare, and the substance responsible is not necessarily present in the living animal. It may well be an artefact. If, however, it is necessary to analyse a material like whale liver oil, the extraction of the "non-sap." by means of 83% alcohol is essential. Chromatographic separations give similar results but are less easily carried out quantitatively. The normal conversion factor should perhaps be used for the fractions soluble in 83% alcohol. Fairly frequent biological assays on anomalous products like whale liver oil are still necessary, although empirical conversion factors can be used for similarly processed batches.

The assay of butter for carotene and vitamin A

Morton and Heilbron (*Biochem. J.*, 1930, **24**, 870) showed that the absorption bands of carotene were clearly exhibited in the non-saponifiable fraction of butter, and from their intensities they were able to calculate the carotene content. The presence of vitamin A was shown by the colour test and the 325mμ absorption band, and the two substances, carotene and

vitamin A, could be determined with fair accuracy. Apart from the intrinsic interest of such results, the method gained additional significance when it was shown that the inclusion in the winter diet of cows of a material rich in carotene (*i.e.* dried grass) reacts very favourably on the carotene and vitamin A content of the butter (Watson, Drummond, Heilbron and Morton, *Empire J. Exp. Agric.*, 1933, **1**, 68; Gillam, Heilbron, Morton, Bishop and Drummond, *Biochem. J.*, 1933, **27**, 878; Booth, Kon, Dann and Moore, *ibid.*, 1933, **27**, 1189).

Gillam (*ibid.*, 1934, **28**, 79) improved the method of assay:

10og. of butter of known moisture content is saponified, using a slight excess of 20% alcoholic KOH (30 mins. under reflux), and the product is treated with 3 volumes of water. The solution is then extracted several times with freshly redistilled ether and the bulked extract is washed repeatedly with water. The solvent is then removed and the last traces of water are eliminated by "blowing" with absolute alcohol and N_2 (see p. 88). The residue is dissolved in pure $CHCl_3$ and the volume is made up to 10 or 20 c.c. The intensity of absorption at 455-460mμ is now determined, using a visual spectrophotometer or an equivalent colorimeter. $E^{1\%}_{1cm.}$ (butter) at 455–460mμ is then calculated and the absorption due to carotene is taken to be 94% of this value. The percentage of carotene in the butter is deduced from the fact that $E^{1\%}_{1cm.}$ 458mμ for pure carotene in $CHCl_3$ is 2,200. The result is expressed in mg. carotene/10og dry butter fat. The figure 94% is taken because separate experiments on a large number of butters showed that the total carotenoids could be separated by the phase test (partition between petrol ether and 90% methyl alcohol) into carotene and "xanthophyll" fractions. It is found that about 6% of the total colour is due to xanthophylls, and departures from the mean in individual samples do not appreciably exceed the experimental error. Gillam therefore eliminated the phase separation.

The vitamin A content of the non-saponifiable fraction can be obtained in two ways: (*a*) at 325mμ the total absorption represents the summation of contributions due to the vitamin and to the carotenoids; from the data for pure carotene and xanthophyll it is found that the visible absorption at 455–460mμ excited by these carotenoids is 6·5 times their absorption at 328mμ; if therefore $E^{1\%}_{1cm.}$ 455–460mμ is divided by 6·5 and the resulting figure subtracted from the gross absorption at 325mμ, it is possible to introduce a correction which will give the net absorption due to the vitamin. By using the conversion factor $E^{1\%}_{1cm.}$328mμ, $1 = 1600$, the potency in respect of vitamin A can be obtained in I.U./g.

(*b*) The $SbCl_3$ colour test for vitamin A $E^{1\%}_{1cm.}$ 620mμ, 6,000 is very much more intense than that of carotene $E^{1\%}_{1cm.}$ 585mμ, 420. It can therefore be used for determining the vitamin and a correction for the carotenoid contribution may be made. Butter 'non-saps.' sometimes contain colour-test inhibitors, and some workers find that the use of traces of bromine in the reagent overcomes this difficulty. In the writer's laboratory, the

colour test on properly prepared "non-saps." has proved quite trustworthy (see Morton, Lord and Goodwin, *J.S.C.I.*, 1941, **60**, 310).

Example. Stall-fed cows.

Butter from	mg. per 100g. butter fat carotene	xanthophyll	Vitamin A	Total potency I.U /g.
Cows fed hay - -	0·272	0·0227	0·42	9·7
Cows fed dried grass -	0·60	0·029	0·72	18·0

Baumann and Steenbock (*J. Biol. Chem.*, 1933, **101**, 547) suggested an alternative procedure: The butter is melted at 55° and decanted from brine and curd on to a cotton filter, and the intensity of absorption at 485 and 460mμ determined spectrophotometrically on the unsaponified clear butter oil at 30°. This is compared with the intensity shown by a standard solution of carotene in refined cottonseed oil also at 30°. (The molecular extinction coefficient of carotene varies from solvent to solvent; the validity of the cottonseed oil comparison has been checked by adding a known weight of carotene to butter fat.) As an example, winter butter contained 0·0022mg. carotene/gm. whilst summer butter contained 0·0062 mg./gm. The total pigment can be obtained by three extractions of the saponified butter with ether, followed by evaporation of solvent in complete absence of air. The residue is taken up in petrol ether, washed three times with an equivalent quantity of 92% methyl alcohol, 95% of the total pigment remains in solution in the petrol ether, so that only 5% of the colouring matter is xanthophylloid.

In order to estimate vitamin A, 15 gm. of butter are refluxed in a stream of nitrogen for half an hour with 125 c.c. of aldehyde-free 12% alcoholic potash (freshly prepared), 125 c.c. of water are then added, and the mixture cooled to 4°, whereupon 150 c.c. of ether are added, followed by 500 c.c. of cold water. The ethereal layer is drawn off and the aqueous alcoholic fraction is extracted three times with successive additional portions of 50 c.c. ether. The combined ethereal extract is washed repeatedly with water, dried over sodium sulphate and the ether removed by evaporation under reduced pressure in a current of nitrogen. The residue is dissolved in 15 c.c. of hot methyl alcohol and left to crystallise at −72° for several hours, using a mixture of solid carbon dioxide and acetone. The cold solution is filtered, washed with cold methyl alcohol and made up to such a volume that $\log I_0/I$ for the cell used (1 cm. or 2 cm.) is about 1·0.

Steenbock and Baumann find that the carotene contained in butter accounts for some 15% of the total growth-promoting effect. The work of Shrewsbury and Kraybill (*Jour. Biol. Chem.*, 1933, **102**, 701) provides emphasis for some of the points already made. Thus the variability of the molecular extinction coefficient from solvent to solvent is shown by adding 0·4–4·0 mg. carotene to 100 gm. butter and comparing

the intensity of absorption with equivalent solutions, using petrol ether. Evaluations of carotene content must be made on the basis of additions of known weights of carotene to strictly comparable solvents, otherwise large errors may occur—if, for instance, oily solutions are balanced against petrol-ether solutions.

It has been found that a deep-yellow butter contains 1·2mg. carotene/100gm. butter
a yellow butter ,, 0·8 ,, ,, ,,
a very pale-yellow butter 0·15mg./100gm.

If carotene is added to butter fat which has been decolorised with charcoal, rapid fading of the pigment occurs. Untreated butter fat contains natural protective substances (destroyed or removed by charcoal) which inhibit this spontaneous decomposition. A trace of hydroquinone acts as an artificial anti-oxidant.

It is fortunate that the carotene of butter consists almost entirely of β-carotene (Gillam and El Ridi, *Biochem. J.*, 1937, **31**, 251), because if α- and β-carotene had been present in variable amounts, as was at one time thought (Gillam and Heilbron, *ibid.*, 1935, **29**, 834), the analysis would have suffered much in respect of precision. Chromatographic investigations (Gillam and El Ridi, *ibid.*, 1936, **30**, 1735) revealed the existence of isomerides of α- and β-carotene which were later found to be artefacts (Zechmeister and Tuzson, *ibid.*, 1938, **32**, 1305; Carter and Gillam, *ibid.*, 1939, **33**, 1325). The carotenes, lycopene and kryptoxanthin all undergo spontaneous isomerisation, and equilibria between opposing reactions are set up. From this work it becomes more than ever clear that in all analyses involving carotenoids: (*a*) the time occupied in manipulation should be reduced to the minimum, (*b*) concentration of solutions should be carried out at low temperatures, and (*c*) concentrates and extracts should be stored at 0°. The determination of vitamin A in fortified margarine is described in an authoritative paper by Edisbury (*Analyst*, 1940, **65**, 484).

The Assay of blood serum for vitamin A and carotenoids

Eekelen (*Acta Brev. Neerland.*, 1931, **1**, 3) took 10 c.c. of serum, precipitated the proteins by addition of alcohol, and, after centrifuging, extracted the precipitate by means of light petroleum. Carotene was estimated in the extract by means of the Zeiss Step-Photometer. The solvent was then removed, and the extract taken up in chloroform and tested by means of the Carr-Price $SbCl_3$ reagent. Carotene was absent from the blood sera of rabbits, dogs and pigs, but vitamin A was shown to be present by the appearance of an absorption band at 610mμ (0·5–2·0 I.U./10 c.c. serum). Cow sera showed the presence of both carotene and vitamin A and human blood sera were similar. Gillam and El Ridi (*Biochem. J.*, 1935, **29**, 2465) subjected blood sera from cattle to direct saponification, and established the

presence of carotene, vitamin A and lutein. They also found that the average carotene and vitamin A contents of the sera of cows were consistently higher than those of bulls, in both winter and summer, whilst the average values for the sera of cattle in winter were definitely lower than the corresponding ones obtained during the spring and summer. (Their paper also gives references to earlier work.)

100 c.c. blood serum were saponified by refluxing for 1 hour with 60% aqueous KOH(10 c c). 30 c.c of alcohol were added to the cold solution which was extracted with 4 portions of 50 c c redistilled ether. After removal of solvent the dried residue (alcohol and N_2) is made up to known volume ($CHCl_3$) and assayed spectrophotometrically for carotene and vitamin A

(See also Estimation of Vitamin A and carotene in human blood, Yudkin, *Biochem. J.*, 1941, **35**, 551.)

The determination of carotene in dried grass and similar products

The production of artificially-dried grass is now a considerable and growing industry. The merits of the material as a winter feeding-stuff are proved, and it has become necessary to have an agreed method of analysis. Although dried grass is richer in protein than hay, it is bought and sold to some extent on the basis of its carotene content. The analytical problem consists in (*a*) obtaining a complete extraction of the pigment, (*b*) separating it from the " xanthophylls ", which appear to have no great significance in nutrition, and (*c*) determining the carotene colorimetrically in solution, without attempting to isolate it.

Most of the methods in use depend on Willstatter's work with Stoll on the pigments of green leaves. The chlorophylls readily lose two ester groups on saponification and yield the green, water-soluble salts *chlorophyllines*; the alkali treatment causes disintegration of the fibre, rendering quite easy complete extraction of the carotenoids by means of organic solvents immiscible with water. The hydroxylated carotenoids are separated from the hydrocarbons by partition between petrol ether and aqueous alcohol, the petrol retaining only the carotene.

Guilbert (*Ind. and Eng. Chem., Anal. Ed*, 1934, **6**, 452; *J Nutrition*, 1935, **10**, 45) took a weighed portion of meal and refluxed it with 25% KOH in methyl alcohol for ½-1 hour. Ether was added to the cooled mixture, the solution decanted off and the residue re-extracted with ether until the decanted liquor was practically colourless. Water was then added to the combined extracts with gentle agitation, and when a good separation was obtained the green lower layer was run off, using a separating funnel The yellow ethereal layer was carefully washed with water several times and then dried over anhydrous Na_2SO_4. The ether was removed completely, preferably in a current of N_2, and the residue taken up in petrol ether The xanthophyll was removed by shaking the petrol solution with aqueous methyl alcohol (10 vols pure methyl alcohol plus 10 vols. distilled water measured separately; specific gravity 0·830 at 15°) The first alcoholic extract could be re-extracted with petrol, but this was not usually worth while. The petrol layer was repeatedly extracted with the aqueous alcohol until the latter removed no more colour. The carotene in the petrol was determined at this stage.

Ijdo (*Biochem. J.*, 1936, **30**, 2307) and Peterson, Hughes and Free-

man (*Ind. and Eng. Chem., Anal. Ed.*, 1937, **9**, 71) extracted the carotenoids directly from the saponification liquor by means of petrol ether. There is, however, a real danger that the saving of time which results from omitting the ether extraction may be more than counterbalanced by the formation of emulsions. Buxton and Dombrow (*ibid.*, 1938, **10**, 262) recommend the use of heptane as extractant. Aqueous saponification was used by Ferguson and Bishop (*Analyst*, 1936, **61**, 515), and by Moon (*J.S.C.I.*, 1938, **57T**, 457 ; *J. Agr. Sci.*, 1939, **29**, 295). After a thorough saponification, the product was filtered at the pump and the residue extracted.

In the partition of carotene and xanthophyll, the aqueous methyl alcohol must be of the specified strength. An alternative is a partition between Skellysolve benzine (*b.p.* 65–75°) and a solution of diacetone (acetone-free), 100 vols., and water, 6 vols. (Hengsted, Porter and Peterson (*Ind. and Eng. Chem., Anal. Ed.*, 1939, **11**, 256).

As far as the writer can judge, the real difficulty in the analysis is in the colorimetry. The Lovibond Tintometer, the Duboscq Colorimeter and the Klett Colorimeter have all been used and a solution of potassium dichromate used as the matching solution on the basis that a 0·025% solution is visually equivalent to a solution of carotene containing 0·158 mg. of β-carotene per 100 c.c. Photo-electric colorimeters or absorptiometers are preferable to the more subjective methods of colour matching. Spectrophotometry with an instrument of the Hilger-Nutting type gives results of satisfactory accuracy. The main carotene maximum in petrol ether occurs at 450mμ, and the intensity of absorption is near $E^{1\%}_{1cm.}$ 2500. Buxton and Dombrow (*loc. cit.*) give the value 2380 in heptane.

The object of the carotene estimation is of course the evaluation of provitamin A potency, and it is fortunate that the carotene of grasses is almost entirely β-carotene. As α-carotene possesses only half the potency of β-carotene a substantial heterogeneity would reduce the value of the assay. In studying palm oil, carrots and yellow maize, where a considerable part of the provitamin activity is due to α-carotene or kryptoxanthin, it is necessary to resort to chromatographic analysis on a micro scale. With a little experience, excellent results can be obtained, but it is advisable to consult the latest literature concerning technique and certain necessary precautions. A detailed account of the methods already published would in all probability be rendered obsolete very quickly as there is much activity in the field.

[Note added Sept., 1941].

See *Analyst*, 1941, **66**, 334, for a Report by a committee of the Grass Driers' Association on carotene estimation. This refers to a valuable paper by Seaber (*ibid.*, **65**, 266), and the final recommendations are based on much experience The Report cannot profitably be abbreviated and should be consulted in the original.

CHAPTER IV

VITAMIN E AND ANTI-OXIDANTS

" The most striking function of vitamin E is to provide for a normal gestation in a pregnant rat, in that it prevents the resorption of the embryo which invariably occurs in its absence " (Olcott and Mattill).

" Antioxidants are substances which when present in minute amounts delay or prevent the autoxidation of fats and inhibit the development of rancidity."

The two concepts—an anti-sterility vitamin and an anti-oxidant are at first sight very remote from one another. The interplay between researches on these two kinds of activity has been more important than is always recognised, and it is convenient to deal with the two subjects together.

The classical work of Evans and his colleagues (1922 onwards) made it clear that absence from a basal diet of a fat soluble accessory factor caused male rats to become infertile through permanent damage to the germinal epithelium, whilst female rats, although capable of conceiving, failed to carry their young for the full term. The embryos died and underwent resorption. The female reproductive organs had, however, undergone no permanent degeneration, since normal gestations could occur when the rats were restored to a diet containing the missing factor. The earlier work has been authoritatively summarised by Evans (*J. Amer. Med. Assoc.*, 1932, **99**, 469). The oils from wheat germ, rice germ and cottonseed were good sources of the vitamin and the first stage of the chemical work consisted in preparing concentrates by elimination of sterols and other substances from the non-saponifiable fractions. It was not easy to control the fractionations because bio-assays were tedious and only semi-quantitative, moreover every attempt to attach a spectroscopic or other " label " to the active principle gave equivocal results. The first crystalline derivatives were obtained as allophanates by treating rich concentrates with cyanic acid. From the recrystallised products two potent alcohols α- and *β-tocopherol* were regenerated, and could be esterified, the esters recrystallised and the alcohols recovered unchanged in potency. Larger quantities of the tocopherols provided material for degradation, and pyrolysis of the α-form gave a crystalline sublimate identified as durohydroquinone: The properties of α-tocopherol were consistent with a chroman structure with a C_{16} side chain. The β-tocopherol

on pyrolytic decomposition gave *pseudo*-cumohydroquinone. Synthesis of the tocopherols soon followed by the condensation of *pseudo*-cumohydroquinone or xylohydroquinones with phytol. Some doubt remained whether a coumaran or a chroman structure was correct, but there is now general agreement in favour of the latter.

Much work has been devoted to the part played by the tocopherols in reproduction and their use in preventing sterility in domesticated animals. Encouraging results have been obtained in dealing with recurrent abortion in women, but the interpretation of clinical experience in this field needs great care. Mattill (*J. Amer. Med. Ass.*, 1938, **110**, 1831) has recently reviewed this aspect of the subject. The symposium held in London in April 1939 (Vitamin E, edited by Bacharach and Drummond, Society of Chemical Industry, London) has been reprinted as a separate volume.*

Wheat germ oil is one of the best sources of the tocopherols, containing perhaps 1 part in 2000; it is also very effective in protecting other fats from becoming rancid. In working up cereal germ oils, the vitamin E and anti-oxygenic activities are accumulated in the same fractions, and in fact the synthetic tocopherols are also powerful "inhibitols". There are, however, anti-oxidants other than vitamins E, and Olcott and Mattill quite early succeeded in isolating a crystalline inhibitol from lettuce (*J. Biol. Chem.*, 1931, **93**, 65) which was not vitamin-active. Neither inhibitol activity nor vitamin potency is strictly specific to the tocopherols and there is evidently divergence as well as overlapping between the two phenomena. The overlap appears clearly in the early discovery that vitamin E readily undergoes destruction by oxidation. Thus rations intended to be free from the vitamin are often treated with ferric chloride; alternatively, rancid or almost rancid fat (presumably free from inhibitols) is added.

The usual method of working up wheat germ oil, rice germ oil, cotton-seed oil, etc., has been saponification, followed by removal of sterols from the non-saponifiable fraction by strong cooling of the methyl alcoholic solution. Chromatographic adsorption, high vacuum distillation, and other recognised procedures, gave promising results and some very potent preparations were obtained. It is now known, as a result of the work of Moss and Drummond (*Biochem. J.*, 1938, **32**, 1953) that considerable destruction of vitamin occurs in the alkali saponification. This loss can be avoided by passing the unsaponified wheat germ oil through a column of alumina, whereby most of the tocopherol (which surprisingly occurs largely as the unesterified alcohol) is adsorbed. The eluate subsequently

* L. I. Smith (*Chem. Reviews*, 1940, **27**, 287–329) gives a fully documented summary of the chemistry of vitamin E. The absorption curves in this section are, with one exception, after Webb *et al.* (*J Org. Chem.*, 1939, **4**, 389).

obtained is greatly enriched in tocopherol and on saponification yields a more potent concentrate, or it may even be treated at once with cyanic acid to form allophanates. This discovery unfortunately came rather too late to influence the main research.

It was very difficult to establish criteria of purity for the highly potent sterol-free oils obtained after selective adsorption, and although absorption maxima were consistently observed at 294mμ by various workers, the difficulty of quantitative bio-assays left the position obscure. No certainty could be reached concerning any property of the vitamin other than that resting on the animal test. The non-saponifiable fractions contained carotenoids, various sterols and higher alcohols, and β-amyrin. After eliminating most of these, the residues were anti-oxygenic as well as active in restoring fertility. Acetylation and benzoylation proved the presence of a hydroxyl group, and the esters retained their vitamin E activity but no longer functioned as inhibitols. Drummond, Singer and MacWalter (*Biochem. J.*, 1935, **29**, 456) obtained exceedingly rich concentrates which now seem to have consisted very largely of the tocopherols. It appeared that the molecules of the active principle contained two or three double bonds and two oxygen atoms, only one of which could be acetylated.

Somewhat crude concentrates of vitamin E had been examined spectroscopically by Bowden and Moore (*Nature*, 1933, **131**, 512; **132**, 423), and by Morton and Edisbury (*ibid.*, 1933, **131**, 618), and by Olcott (*J. Biol. Chem.*, 1934, **107**, 471, 685). The results were equivocal although it was tempting to associate a maximum at 294mμ with the vitamin (*cf.* Martin, Moore, Schmidt and Bowden, *Nature*, 1934, **134**, 214). The objection to this is illustrated by Olcott's experience:

Preparation	Vitamin E minimum dose for a rat mg.	$E^{1\%}_{1cm.}$ 294mμ	anti-oxygenic index
1	4	70	4–7
2	30	90	5–8
3	3	100	5–8
4	10	65	5–8
5	2	70	5–8

Even allowing for experimental error, it seemed as if the band at 294mμ was more plausibly to be ascribed to an inhibitol than to vitamin E, and Olcott's caution in refusing to assume otherwise did not lack justification.

The fundamental importance of obtaining the vitamin, or a derivative, in a crystalline form, was now more than ever manifest and the success of Evans and the Emersons (*J. Biol. Chem.*, 1936, **113**, 319) in this direction

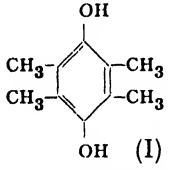
was a decisive step forward. They found that cyanic acid attacked the free hydroxyls to form allophanates which could be crystallised:

$$2HCNO + ROH \longrightarrow H_2NCONHCOOR$$

Two products melting at 160° and 146° respectively were obtained. From these, two alcohols, α-tocopherol $C_{29}H_{50}O_2$ and β-tocopherol $C_{28}H_{48}O_2$, were obtained and found to be active at doses of 3 and 5–8mg. respectively. They could be esterified and the esters recrystallised. The regenerated unchanged alcohols were pale oils showing the characteristic absorption maximum at 294-298mμ which could now be definitely associated with vitamin E.

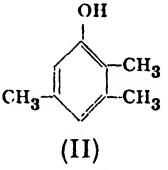
The allophanates made it possible to obtain sufficient α- and β-tocopherol for degradation on a reasonable scale, especially when it was found that α-tocopherol and a third substance γ-tocopherol could be isolated from cottonseed oil. An apparent discrepancy remained in that some concentrates showed extinction coefficients greater than pure α- or β-tocopherol, but many difficulties were resolved when Olcott and Emerson (*J. Amer. Chem. Soc.*, 1937, **59**, 1008) found that all three tocopherols were powerfully anti-oxygenic, the γ-compound being the most effective, whilst α-tocopherol was definitely the most potent anti-sterility agent.

Various groups of workers attacked the problem of structure and a substantial contribution came from Fernholz (*J. Amer. Chem. Soc.*, 1937, **59**, 1154; 1938, **60**, 700). His evidence may be summarised:

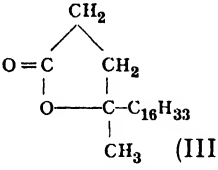


(I)

in good yield.

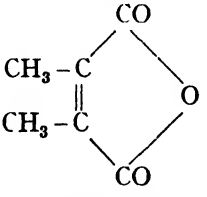


(II)



(III)

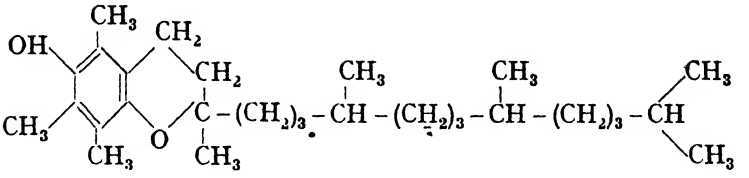
(*a*) Esterification indicated one hydroxyl group, (*b*) heating to 350° gave durohydroquinone (I), (*c*) heating with hydriodic acid gave *pseudo*-cumenol (II), (*d*) mild oxidation by means of chromic acid gave a C_{21} lactone (III), the free hydroxy acid being readily relactonised, so that a tertiary hydroxyl (hydroxy acid) had to be postulated. More vigorous oxidation yielded dimethyl maleic anhydride,



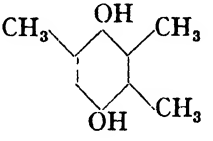
diacetyl, acetone, a $C_{16}H_{33}.COCH_3$ ketone and a C_{16} acid, $C_{15}H_{31}.COOH$.

(*e*) Absorption spectra indicated a phenolic hydroxyl.

Fernholz correctly ascribed to α-tocopherol the chroman structure

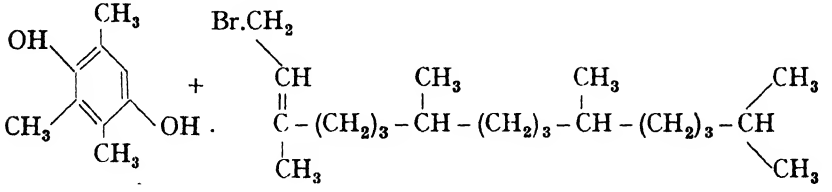


although a coumaran structure was possible. This residual uncertainty was resolved by John (*Z. physiol. Chem.*, 1939, **257**, 173) by arguments based on absorption spectra. The same author (*ibid.*, 1937, **250**, 11) had earlier obtained *pseudo*-cumohydroquinone (instead of durohydroquinone) from β-tocopherol. He had at first suspected that the vitamin was a simple mono ether of *pseudo*-cumohydroquinone, but, like Fernholz, he



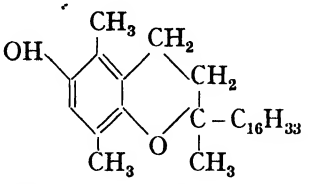
rejected this idea on spectroscopic evidence. Thus the mono n-dodecyl ether of durohydroquinone shows λ_{max} 280–285mμ, $E^{1\%}_{1cm}$ 50 as against $\lambda_{max.}$ 298mμ, $E^{1\%}_{1cm.}$ 70 for α-tocopherol.

The first synthesis was achieved by Karrer, Fritzsche, Ringier and Salomon (*Helv. chim. Acta*, 1938, **21**, 520, 939):



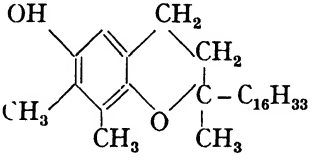
Psuedo-cumohydroquinone reacts with phytyl bromide in the presence of anhydrous zinc chloride and petrol at 80°, to give racemic *dl*-α-tocopherol smoothly and in almost quantitative yield. The allophanate, *m.p.* 172°, was recrystallised and the pure alcohol regenerated. A product identical with natural α-tocopherol was obtained by Bergel, Copping, Jacob, Todd and Work (*J. Chem. Soc.*, 1938, 1302) from phytol and *pseudo*-cumohydroquinone, and from the same quinol and phytadiene by L. Smith, Ungnade and Pritchard (*Science*, 1938, **88**, 37). In the rat test, 2-3mg. of synthetic α-tocopherol or its acetate is sufficient to provide for a normal gestation. The *d*- and *l*-forms do not differ recognisably in potency.

Later (Karrer and Fritzsche, *Helv. chim. Acta*, 1938, **21**, 1234, 1622, **22**, 260, 654, 661 ; Bergel and Todd, *J. Chem. Soc.*, 1939, 542), the three isomeric xylohydroquinones were condensed with phytol (using formic acid or alternatively the monobenzoates of the xylohydroquinones). The compound



proved identical with β-tocopherol

and



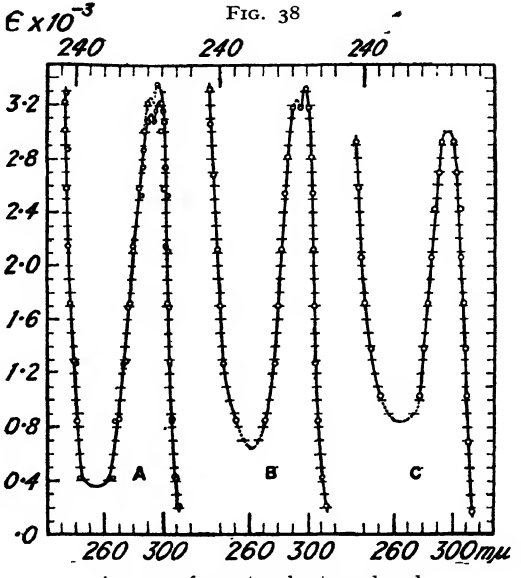
with γ-tocopherol

At this stage it became possible to study the specificity of the structure-potency relationship, and a great deal of work can be briefly summarised :

	active dose
Chromans	
2, 2-diethyl or dibutylchroman	100 mg.
2, 5, 7, 8-tetramethylchroman	30 ,,
2, 5, 7, 8-tetramethyl, 6-hydroxychroman	100 ,,
o-xylotocopherol (*dl-γ*, *i.e.* *2$C_{16}H_{33}$, 2, 7, 8-trimethyl, 6-hydroxychroman)	6–8 ,,
m-xylotocopherol	6–8 ,,
p-xylotocopherol (*dl-β*, *i.e.* *2, $C_{16}H_{33}$, 2, 5, 8-trimethyl, 6-hydroxychroman)	6–8 ,,
α-tocopherol (*i.e.* *2, $C_{16}H_{33}$, 2, 5, 7, 8-tetramethyl, 6-hydroxychroman	2–3 ,,
α-tocopherolacetate (*dl-*)	1–2 ,,
Coumarans	
2, 2-dimethyl-coumaran	50 ,,
2, 2, 7-trimethyl-coumaran	100 ,,
2, 2-di-n-butyl-coumaran	100 ,,
3-carbethoxy, 5, 7, 8-trimethyl, 6-hydroxy-coumarin	20 ,,
o-allylphenol	50 ,,
p-amino-o-allylphenol	50 ,,
2, 3-dimethylhydroquinone / durohydroquinone / duroquinone (also various ethers)	100 ,,

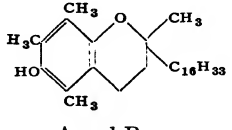
* See p. 107

FIG. 38

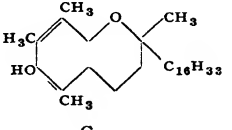


A, curve for natural α-tocopherol
B, curve for synthetic *dl*-α-tocopherol
C, curve for m-xylotocopherol

After Webb *et al.* See also Smith *loc. cit.*

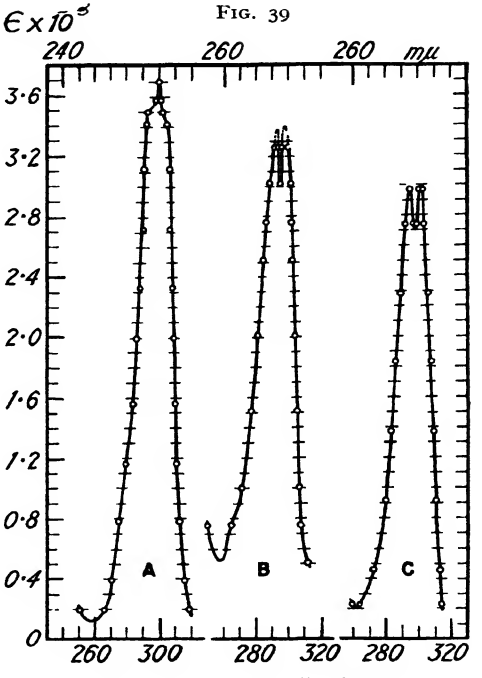


A and B

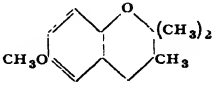


C

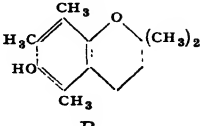
FIG. 39



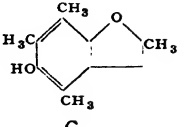
Curves for " model " substances



A



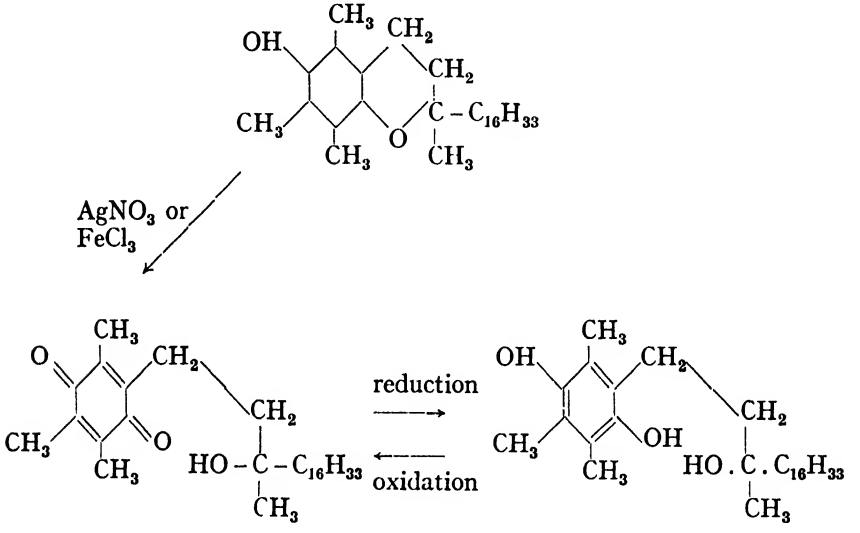
B



C

Over 150 compounds have been tested and about forty found to be active, when the level of dosage is high enough. It is evident that high potency (3–10 mg. dose) is restricted to very few tocopherols, but if the very high doses of 50–100 mg. are used, the response is far from specific.

The oxidation products of the tocopherols are important not only because they may be concerned in physiological mechanisms, but also because of the inhibitol effect. John (*Z. physiol. Chem.*, 1937, **250**, 11) isolated a well-crystallised quinone as follows:



The product is easily reduced to α-tocopherylhydroquinone, which reverts to the quinone in contact with air, and with strong acids loses a molecule of water to regenerate α-tocopherol. The quinone is biologically inactive even at a dose level of 30 mg., a fact which militates against the idea that the compound enters into the utilisation of the vitamin in the body though an oxidation-reduction process.

The spectroscopic study of materials containing tocopherols has led to data which cannot yet be interpreted entirely satisfactorily. The tocopherols show $\lambda_{max.}$ *ca*294 mμ, $E^{1\%}_{1cm.}$ 100, so that for a wheat germ oil containing 0·5% of vitamin E (*cf.* Karrer and Keller, *Helv. chim. Acta*, 1938, **21**, 1161) the absorption due to the vitamin will be $E^{1\%}_{1cm.}$ 294mμ 0·5. A typical wheat germ oil may show $\lambda_{max.}$ 272mμ, $E^{1\%}_{1cm.}$ *ca* 2·0, so that 75% at least of the absorption is irrelevant. This precludes direct spectroscopic assays. In the non-saponifiable fractions as normally obtained, the toco-

pherol content has diminished owing to the deleterious action of alkali. The sterol portion shows the ergosterol-like bands, and the non-crystallisable portion shows maxima at 480, 446, 421, 291, 271, 256·5 mμ and some fractions show marked bands near 280 and 253mμ. Carotenoids, steroids and decomposition products occur, and mask the tocopherol absorption, and it is only after chromatographic adsorption or preparation of allophanates that the 294mμ absorption band can be used as a quantitative criterion of potency. It is not yet clear to what extent the preliminary adsorption used by Moss and Drummond will affect the method, although a 10–15-fold increase in potency may well diminish the importance of irrelevant absorption. For rich concentrates and synthetic preparations, spectroscopic control is very suitable. Care should, however, be taken to use the correct wave-length since $\lambda_{max.}$ for synthetic *dl*-α-tocopherol acetate (in alcohol) occurs at 285mμ. A useful method for determining tocopherols, based on a potentiometric titration involving the use of gold chloride as oxidising agent has been worked out by Karrer and Keller (*loc. cit.*). Carotenoids interfere and must be allowed for, but the method works well for quite small amounts of tocopherols. Emmerie and Engel (*Rec. Trav. Chim.*, 1938, **57**, 1351; 1937, **58**, 283) oxidise the tocopherols in alcoholic solution by means of ferric chloride and estimate the ferrous iron by the αα′-dipyridyl method. The intensity of colour may be measured spectroscopically or by means of the Zeiss Pulfrich Step-Photometer. Carotenoids again interfere, but may be removed prior to oxidation by filtering the concentrate in benzene solution through a layer of Floridin XS earth. The method is not applicable to tocopherol esters, and as saponification always involves some destruction, it cannot easily be modified to make it so.

Furter and Meyer (*Helv. chim. Acta*, 1939, **22**, 240) have used a colour test based on the oxidation of tocopherols by means of nitric acid to give a deep-red solution. Carotenoids do not in this case interfere, but inactive quinonoid oxidation products (which are often present in oils) enhance the colour. Thus Lester Smith and Bailey (*Symposium*, see p. 104) found in one case a reading of 13 by this method as against 3 by either the gold chloride or the ferric chloride method. The usefulness of the iron salt is illustrated by the data of Emmerie and Engel on blood sera from rats:

	μg. 10 c.c. blood serum
Normal diet	6·4 males; 5·6 females
,, ,, supplemented by wheat germ oil	26 males; 20 females
,, ,, ,, ,, *dl* α-tocopherol	100

The administration of *dl*-α-tocopherol acetate results in an increase in *free* tocopherol in the serum.

John has pointed out that the quinones formed from the tocopherols show at 265mμ a maximum which is five times as intense as the 294mμ

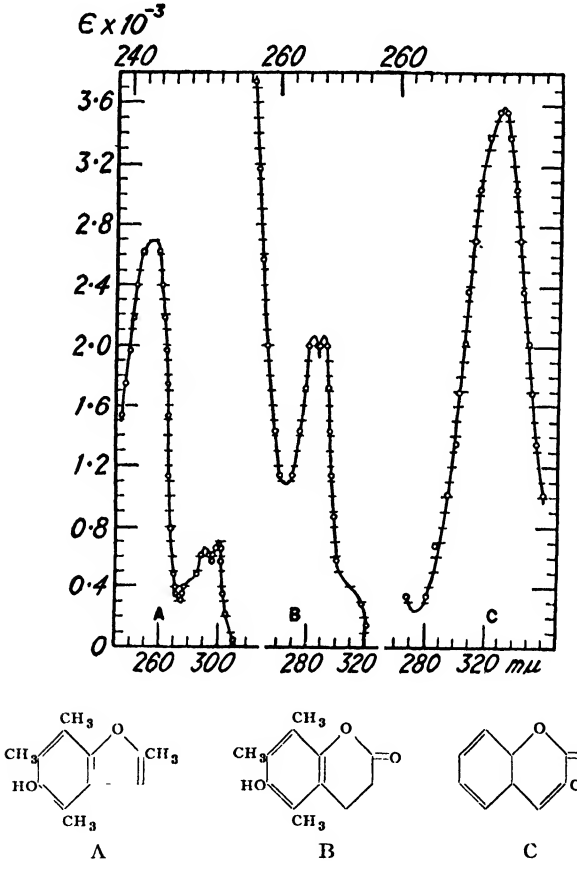


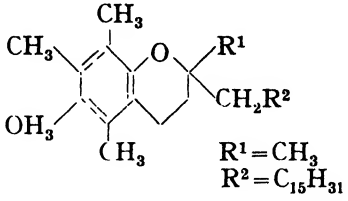
FIG. 40

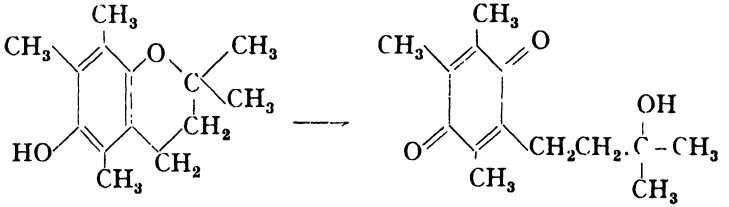
After Webb *et al.*

band, and may perhaps be useful in determining potency. This possibility has not yet been fully explored.

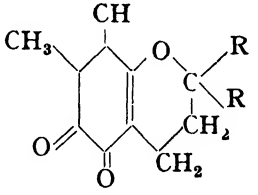
Smith, Irwin and Ungnade (*J. Amer. Chem. Soc.*, 1939, **61**, 2425) studied the oxidation of α-tocopherol by means of ferric chloride, gold chloride,

etc., the products are yellow *p*-quinones both for tocopherol and its homologue 2,2,5,7,8-pentamethyl 6-hydroxy-chroman :

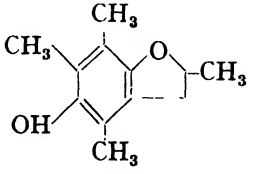


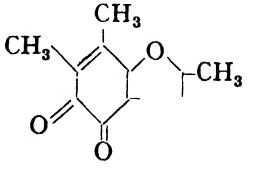


(*cf.* John, Dietzel and Emte, *Z. physiol. Chem.*, 1939, **257**, 173 ; Karrer, Fritzsche and Escher, *Helv. chim. Acta*, 1939, **22**, 661). Silver nitrate acts similarly, but prolonged reaction gives a brilliant red colour like that produced by nitric acid. The method is also applicable to *p*-hydroxy-coumarans and chromans in general (*J. Org. Chem.*, 1939, **4**, 298), since 6-hydroxy-chromans when oxidised by silver nitrate or nitric acid yield red-coloured chroman-5,6-quinones :

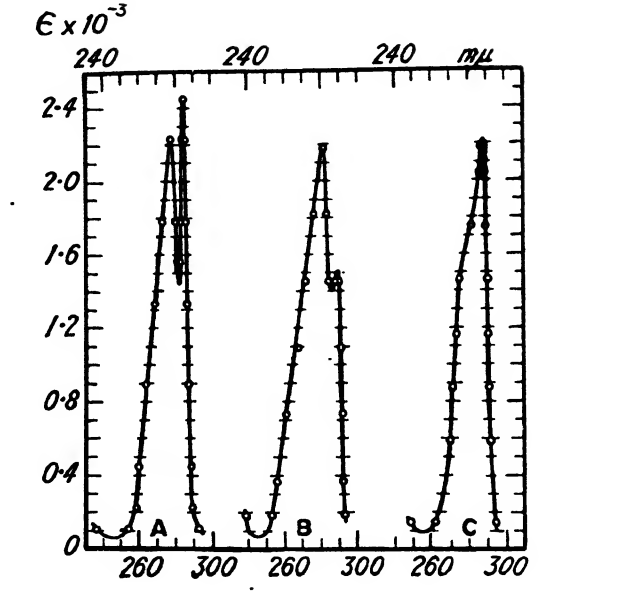


Hydroxy-coumarans of the type shown below are similarly oxidised to red compounds.



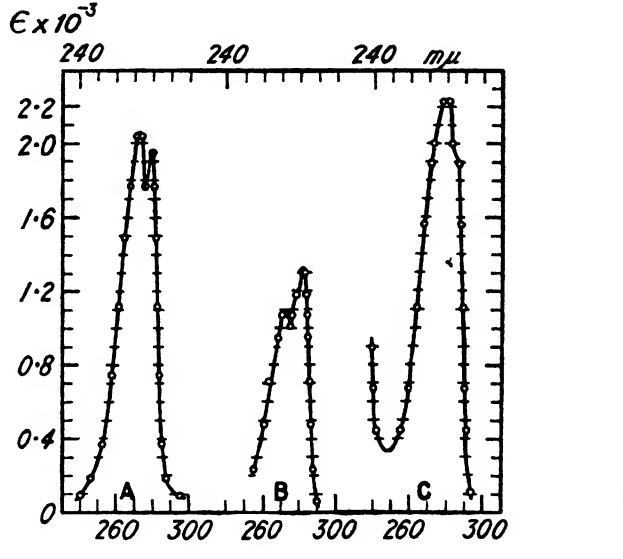


Ungnade and Smith (*J. Org. Chem.*, 1939, **4**, 397) record absorption curves for the solutions obtained in the Furter and Meyer test and give **E** values for the tocopherols ($\lambda_{max.}$ 467mμ). The test applies not only to the tocopherols but to 6-hydroxy-chromans generally, and the spectroscope shows that the colour given by 5-hydroxy-coumarans can be distinguished from that given by 6-hydroxy-chromans.



A B C

FIG. 41



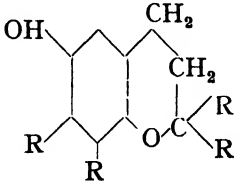
A B C

FIG. 42

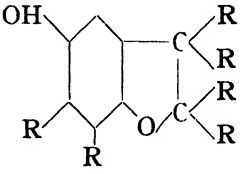
After Webb *et al.*

Spectrographic data

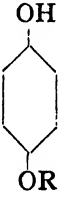
The tocopherol spectra can best be understood by comparing them with those shown by simpler substances. Phenol shows $\lambda_{max.}$ *ca* 275mμ_2, $\epsilon_{max.}$ 2,000; hydroquinone in alcohol has its maximum at 294mμ, $\epsilon_{max.}$ 3,100, whilst for durohydroquinone the corresponding figures are 287mμ and ϵ_{max} 3,400. The durohydroquinone mono-ethers exhibit a decrease in $\epsilon_{max.}$ to *ca* 2000 and a displacement of $\lambda_{max.}$ to 280mμ. From the point of view of the theory of chromophoric groups



and



are essentially the same as



and it turns out that 6 hydroxy-chromans, 5 hydroxy-coumarans, and α- and β-tocopherols are almost identical as regards their absorption spectra both before and after esterification (Bergel, Jacob, Todd and Work, *Nature*, 1938, **142**, 36; John, Dietzel and Emte, *Z. physiol. Chem.*, 1939, **257**, 173); *cf.* also 2,5,7,8-tetramethyl 6-hydroxy-chroman, $\lambda_{max.}$ 298mμ, log ϵ 4·25, 2-methyl, 5-hydroxy-coumaran, $\lambda_{max.}$ 302mμ, log ϵ 3·4 and Figs. 41–43

The ϵ values are dependent to a considerable extent on the substituents. The allophanates exhibit a displacement of the maxima in the direction of shorter wave-lengths, *e.g.*:

	α-tocopherol	α-tocopheryl allophanate
$\lambda_{max.}$	297mμ	282mμ
$\epsilon_{max.}$	3800	1700

John, Dietzel and Emte (*loc. cit.*) were able to show clearly that coumarans and chromans can be differentiated when they form allophanates (Fig. 43).

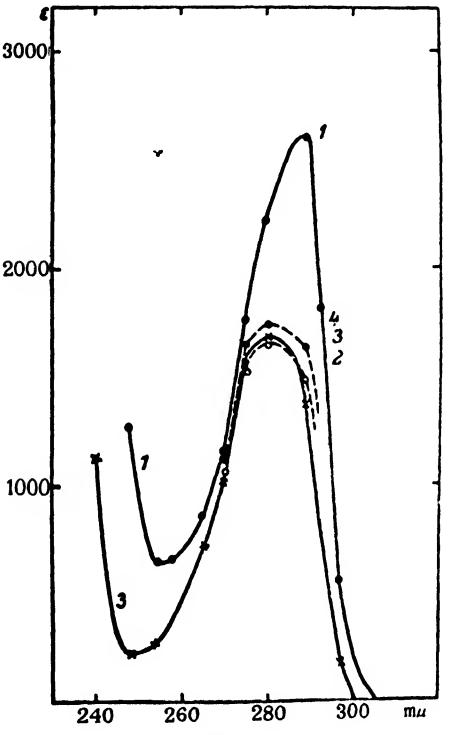


FIG. 43

Allophanates of

1. ●—●—● Tetramethyl-hydroxy-coumaran.
2. ○-·-·-○ Pentamethyl-hydroxy-chroman.
3. × —— × α-Tocopherol.
4. ●-·-·-● Didodecyl-trimethyl-hydroxy-chroman

After John *et al.*

Natural anti-oxidants or inhibitols

The anti-oxidants are of such great actual and potential practical importance that it will not be out of place to discuss them here, particularly since the methods of study applicable to the fat-soluble vitamins are valid in this field also (*cf.* Diemair and Fox, *Angew. Chem.*, 1939, **52**, 484). Among the anti-oxidants of known constitution are quinol, catechol, pyrogallol, monomethyl hydroquinone, phosphoric esters of glycol and glycerol, and many polyhydroxy compounds linked to lipophilic fat residues of high molecular weight, and, as has already been mentioned, the tocopherols. There seems to be little doubt that certain phosphatides are also effective.

Mattill and his colleagues have for some years been trying to isolate and identify the natural anti-oxidants. Olcott and Mattill (*Proc. Iowa*

Acad. Sci., 1931, **38**, 172) isolated from lettuce a compound, $C_{13}H_{14}O_5$, which was powerfully anti-oxygenic. Later, Bradway and Mattill (*J. Amer. Chem. Soc.*, 1934, **56**, 2492) found that the active substances obtained from the seed fats of tomatoes and carrots, and from wheat germ, were different from each other and from that contained in lettuce. Olcott and Mattill (*ibid.*, 1936, **58**, 1627) obtained from wheat germ oil, cotton-seed and palm oil, products superficially resembling vitamin E and showing selective absorption at 294mμ of the kind now associated with the tocopherols. These inhibitols are unsaturated, they contain one hydroxyl group and appear to be nitrogen-free. Hilditch and Dean (*cf.* also Hilditch and Sleightholme, *J. Soc. Chem. Ind.*, 1932, **51**, 39T) obtained from the non-saponifiable fraction of linseed oil a syrupy concentrate, of great anti-oxygenic activity, which reduced Tollen's reagent and alkaline permanganate, and readily decolorised a solution of bromine (iodine value 145). The material showed bands at 256, 420, 445 and 475mμ, the absorption spectrum being very similar to that shown by vitamin E concentrates prior to the preparation of allophanates. Green and Hilditch (*ibid.*, 1937, **56**, 23T) obtained a larger yield of the active syrup from linseed oil foots. They further made the important observation that extracted soya bean meal, after digestion with dilute acetic acid, yielded on heating with methyl alcohol a much larger quantity (possibly 2% of the weight of cake) of active syrup. Hilditch and Paul (*ibid.*, 1939, **58**, 21) studied extracted palm kernel, ground nut, soya bean, cottonseed and linseed meals by the following procedure:

(*a*) a preliminary digestion with a 10% solution of acetic acid in methyl or ethyl alcohol was followed by filtration;

(*b*) the alcohol was removed by evaporation under reduced pressure and the residue separated, by addition of acetone, into an inactive insoluble fraction and an active acetone-soluble fraction.

The anti-oxygenic power was assessed by determining the time needed for 10 c.c. of distilled olive oil esters containing 0·02 g. of concentrate to reach a peroxide value of 50 when aerated at 99·5°.

Antioxygenic activity of concentrates.

Extracted meal	%N.	Primary digestion	Acetone soluble Concentrate Yield %	Time (hrs. in test)
Soya bean	7·4	10% acetic acid +ethyl alcohol	4·1	6
Ground nut	8·6		5·0	7
Cottonseed	4·8		3·7	9
Linseed	5·8		5·3	6
Palm kernel	2·9		3·9	6

By using 5% acetic acid and acetone for the primary digestion, smaller yields of more potent concentrates were obtained. The results of many experiments indicated that the products were heterogeneous and the anti-oxygenic activity could not be correlated with any other property. The concentrates showed marked reducing power and gave positive tests for carbohydrates ; they gave only a feeble brown colour with ferric chloride, and contained 0·4–1·0% of combined phosphorus and 1–2% of combined nitrogen. Anti-oxygenic activity was suppressed by treatment with anhydrous hydrogen chloride, but partly restored on cautious neutralisation with sodium methoxide. The inhibitor thus seemed to be a basic compound.

Diemair and Fox (*loc. cit.*) find that by shaking ground oatmeal with twice its weight of petrol ether for about 10 days at room temperature, a clear greenish-yellow oil of high anti-oxidant activity can be obtained if the filtered solution is evaporated under reduced pressure. The oil is dissolved in ice-cold ether, and 5 volumes of acetone, cooled to 0° C., added. A voluminous grey precipitate is obtained which turns reddish when the solvent is removed completely. The precipitate, while still moist, may be dissolved in benzene and reprecipitated with acetone. The precipitation may be repeated twice and is found to result in an oil-soluble material retaining all the anti-oxidant activity. The precipitate gives positive protein reactions (Adamkiewitz, biuret, Millon, xanthoprotein), it reduces Fehling's solution and dissolves in chloroform, benzene and pyridine. The phosphorus nitrogen ratio is 1 : 17·5.

If oatmeal oil in benzene and ether (1 : 4) is treated with ice-cold acetone a phosphatide is thrown out of solution, and there appears to be a close connexion between lecithin and the anti-oxidant. The oxidation-inhibitors appear to occur as lipoid or protein complexes and to lose activity when these are broken down. Much work remains to be done in this field.

CHAPTER V

VITAMIN K

In 1930 Dam (*Biochem. Z.*, **220**, 158) fed hens on a diet of casein, starch, filter paper and salts, with cod liver oil and yeast extract (Marmite) to supply vitamins A and D and the B complex. His immediate object was to ascertain whether cholesterol could be synthesised in the body. The diet was evidently incomplete and post-mortem examinations revealed erosion of the gizzard, and haemorrhage, both subcutaneous and intramuscular. The basal diet caused chicks to become anaemic, the erythrocyte count being less than two-thirds of the normal value. Further study showed that the blood was abnormally long in clotting, and the concept emerged of a fat-soluble vitamin designated K. The criterion of avitaminosis K which lends itself to animal experimentation is a greatly lengthened blood-clotting time, and the test for the occurrence of the vitamin is a normal blood-clotting time in chicks on the basal diet supplemented by the material under study. Rats and guinea pigs do not develop the symptoms of vitamin K deficiency, and human haemophiliacs gain no benefit from vitamin K concentrates. Pig liver fat is a better source of the vitamin than fish liver oil or chick liver fat. Green vegetables, particularly alfalfa, cabbage, spinach and hempseed are also rich in the vitamin.

McFarlane (*Biochem. J.*, 1931, **25**, 358) demonstrated clearly the fat-soluble nature of the vitamin, because chickens on a similar basal diet supplemented by *extracted* fish meal suffered greatly from haemorrhages and many deaths occurred, whereas chickens receiving unextracted meal were quite healthy. Although the symptoms bore some resemblance to those of scurvy, vitamin K could not be replaced by ascorbic acid nor by lemon juice. The prothrombin content of the blood is reduced in avitaminosis K, but the significance of this fact is not clear. The restoration of normal blood-clotting time is a rapid process taking not more than 2–3 days when sufficient vitamin K is supplied.

Almquist (*J. Biol. Chem.*, 1936, **114**, 241) prepared concentrates of vitamin K from the hexane extract of alfalfa. Distillation at very low pressures yielded an active, colourless, liquid fraction, 2 mg. of which proved adequate for 1 kg. of feed. The material was unsaturated and unstable to alcoholic alkali.

At this stage attention was turned to improving the animal method of assay. Although blood-clotting time is not accelerated *in vitro*, intravenous injection of suitable preparations is effective in 4–6 hours, and overdosage does not increase coagulability beyond the normal. Almquist maintains newly hatched chicks on a diet devoid of vitamin K for 12 days; at this point 1 c.c. of ground-nut oil containing 250μg. of test substance is administered by means of a tube into the crop, and the blood-clotting time determined next morning. A biological technique as clear-cut as this makes the problem of isolating the active principle largely a matter of time and resources of personnel and money, and it is not surprising that several groups of workers should have

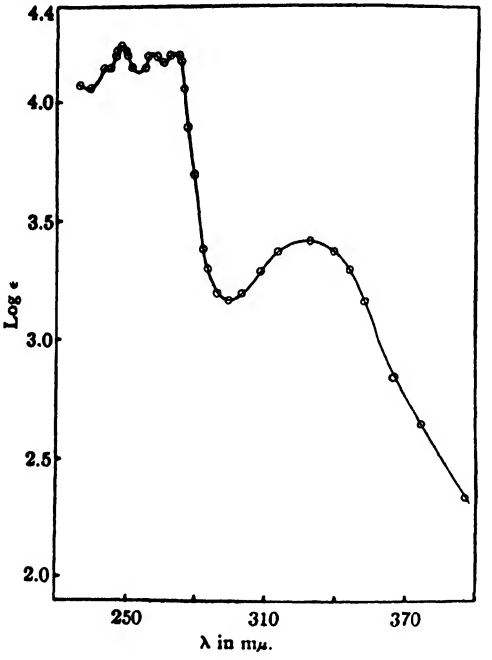


FIG 44

2-Methyl-3-phytyl-1,4-naphthoquinone in alcohol.
After Fieser *et al.*

arrived at the truth almost simultaneously; although much skill and patience had to be shown.

By a combination of selective adsorption and molecular distillation Dam, Geiger, Glavind, Karrer, Karrer, Rothschild and Salomon (*Helv. chim. Acta*, 1939, **22**, 310) obtained a yellow oil, apparently homogeneous and certainly highly potent. The preparation showed absorption maxima at 248, 261, 270 and 328mμ, $E^{1\%}_{1cm.}$ 248mμ, 280 (Fig. 44). On catalytic

hydrogenation only the 328 mμ maximum disappeared, and it was argued that the vitamin K molecule contained two distinct chromophoric units. These authors also described a colour test for vitamin K capable of being used as a quantitative measure of activity.

A few mgms of concentrate, when treated with 1 c.c. of sodium methoxide solution (2–3 g sodium in 50 c c. methyl alcohol) and warmed for a few minutes, develops a transient purple coloration which soon becomes a fairly stable reddish brown. At this stage, partition between petrol and methyl alcohol is used to eliminate interfering colour due to carotenoids. The methyl alcohol layer retains all the colour ascribed to vitamin K.

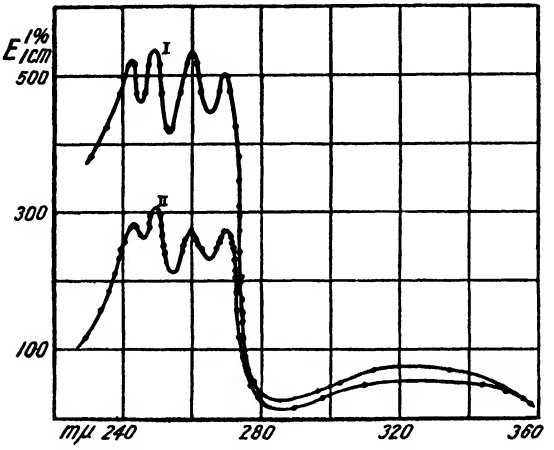


FIG. 45

I vitamin K_1 } in hexane
II vitamin K_2 }

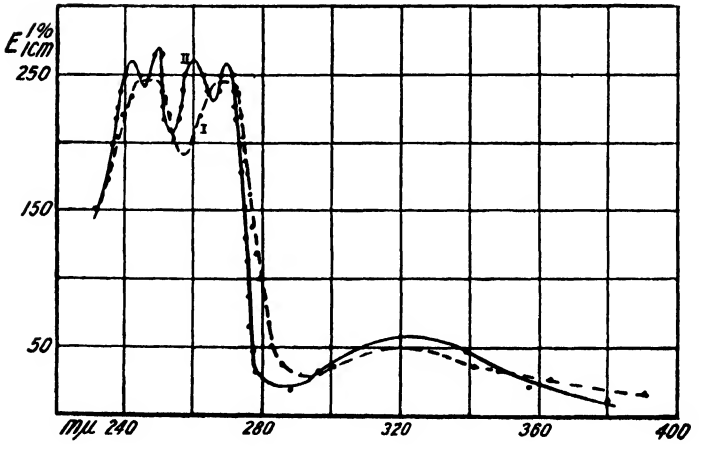


FIG. 46

Vitamin K_2, I in alcohol; II in hexane
After Ewing, Vandenbelt and Kamm

A close parallelism between clotting times and colour-test intensity induced belief in the specificity of the test.

Claims to have isolated in a state of purity two natural products both exhibiting high vitamin K potency, were made early in 1939 by McKee, Binkley, MacCorquodale, Thayer and Doisy (*J. Amer. Chem. Soc.*, 1939, **61**, 1295). Vitamin K_1 from alfalfa, is a light yellow oil, $C_{32}H_{48-50}O_2$, crystallising from alcohol or acetone at low temperatures. The absorption spectrum shows maxima at 243, 248, 261, 270 and 323mμ, $E^{1\%}_{1cm.}$ 248mμ, 540. Vitamin K_2 from putrefied sardine meal is a light yellow solid, *m.p.* 50·5–52°, $C_{40}H_{54-56}O_2$; it shows maxima at 249, 261, 269, 340 and (?) 430mμ. This substance exhibits *ca* 0·6 times the activity of vitamin K_1. The same group of workers subjected both vitamins to reductive acetylation and obtained crystalline products:

dihydrovitamin K_1 diacetate $C_{36}H_{54-56}O_4$, *m.p.* 59°

λ_{max} 230mμ, $E^{1\%}_{1cm.}$ 1250

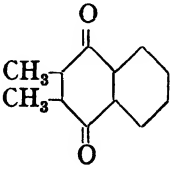
activity 0·5 times that of vitamin K_1

dihydrovitamin K_2 diacetate $C_{44}H_{60-62}O_4$, *m.p.* 57–58°

λ_{max} 232mμ, $E^{1\%}_{1cm.}$ 1250

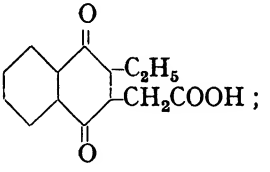
activity 0·3 times that of vitamin K_1.

The pure vitamin K_1, regenerated by treatment of the dihydro-diacetate with methyl magnesium iodide followed by shaking the hydrolysed product with air, showed the normal absorption spectrum and full activity (*J. Amer. Chem. Soc.*, 1939, **61**, 1612). The above indications of a quinonoid nature for the vitamin were supported by the observation published at the same time (*ibid.*, p. 1611), by Almquist and Klose, that phthiocol prepared synthetically and known to possess the structure:

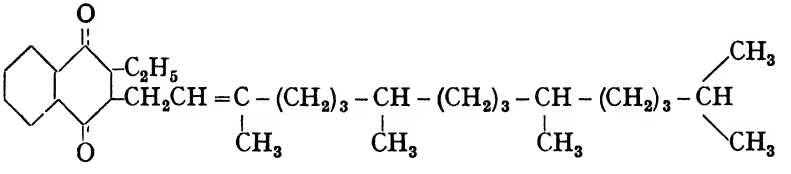


was markedly active in 10 mg. doses. At the same time, Ansbacher, Fernholz and Moore (*ibid.*, 1613) indicated that the sodium methoxide colour test of Dam *et al.* was due, not to vitamin K, but to readily formed

decomposition products. The following month (*ibid.*, p. 1924) Almquist and Klose recorded the fact that 2-methyl 1,4-naphthoquinone and phthiocol monoacetate were more active than phthiocol. Ansbacher and Fernholz confirmed the activity of phthiocol, but found that 2-methyl 1,4-naphthoquinone was several hundred times more active, and practically as potent as vitamin K. With minute doses of the 2-methyl compound the deficiency symptoms disappeared in 24 hours and the chicks doubled their weight in 10 days. A further contribution was also made by Thayer, Cheney, Binkley, MacCorquodale and Doisy (*ibid.*, 1932); they had tested many quinones but had found activity to be confined to 1,4-naphthoquinones and especially those with 2-alkyl groups. The same group of workers had already shown that vitamin K_1 and vitamin K_2 molecules absorbed 8 and 18 atoms of hydrogen respectively on catalytic hydrogenation. In the former, saturation of the 1,4-naphthoquinone could account for 3 mols. of hydrogen so that the remaining double bonds probably occurred in the side chain of K_1 and K_2. Ozonolysis of the reduced vitamin K_1 yielded a ketone 2,6,10-trimethyl pentadecanone-14, indicating a phytyl side chain. Oxidation by means of chromic acid gave phthalic acid and a quinone, almost certainly 2-ethyl, 1,4-naphthoquinone-3-acetic acid:



vitamin K_1 is thus 2-ethyl,3-phytyl,1,4-naphthoquinone:



In the same number of the Journal (*ibid.*, 1925), Fieser, Bowen, Campbell, Fry and Gates pointed out that vitamin K_2 was probably 2,3-difarnesyl, 1,4-naphthoquinone, and that from the point of view of biogenesis the replacement of the phytyl group in the vitamin K_1 of plant origin, by farnesyl (with its relationship to squalene) in the sardine meal, was not very surprising.

Absorption Spectra of Vitamins K_1 and K_2 and related compounds.

In hexane, 1,4-benzoquinone shows an intense band with $\lambda_{max.}$ 241mμ, ϵ_{max} 22,700, with a weak band of small persistence near 290–295mμ, $\epsilon_{max.}$ *ca* 1350. The following table shows the data for 1,4-naphthoquinones:

	$\lambda_{max.}$ A.	log $\epsilon_{max.}$		$\lambda_{max.}$ A.	log $\epsilon_{max.}$
1,4-Naphtho-	2410	4·32	2,3-Dimethyl-1,4-	2430	4·26
quinone (Fig. 47)	2460	4·37	naphthoquinone	2490	4·26
	2510	4·28		2600	4·28
	2560	4·12		2690	4·28
	3300	3·44		3300	3·38
2-Methyl-1,4-	2440	4·29	Vitamin K_1	2430	4·37
naphthoquinone	2500	4·29		2490	4·39
	2630	4·30		2600	4·38
	3340	3·38		2700	4·36
				3250	3·52
2-Ethyl-1,4-	2450	4·31	Vitamin K_2	2430	4·20
naphthoquinone	2500	4·34		2490	4·24
	2550	4·33		2600	4·18
	2670	4·26		2700	4·18
	3300	3·4		3250	3·45

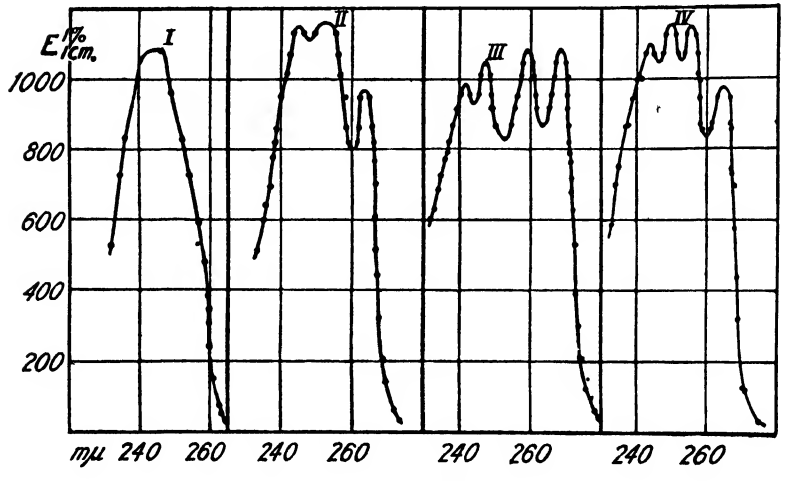
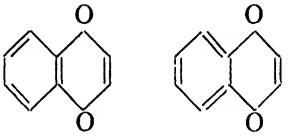


FIG. 47

1,4-Naphthoquinone (curve I).
2-Methyl-1,4-naphthoquinone (curve II).
2,3-Dimethyl-1,4-naphthoquinone (curve III).
2-Ethyl-1,4-naphthoquinone (curve IV) in hexane.
After Ewing *et al.*

In terms of resonance naphthoquinone possesses a hybrid structure, the extreme forms being :



Morton and Stubbs (*J. Chem. Soc.*, 1940, 1347) showed that salicylaldehyde and o-hydroxy-acetophenone were spectroscopically similar, having maxima near 325mμ, log ϵ 3·5, and 255mμ, log ϵ 40, characteristic of the benzenoid chromophore $C_6H_4\langle^{CO\,.\,R.}$ In the simple quinones, weak and resolved absorption (ϵ values 1–20) occurs in the visible. In naphthoquinone (Macbeth, Price and Winsor, *J. Chem. Soc.*, 1935, 325) there is a marked inflexion at 390–460mμ, log ϵ 1·9–1·6 as well as the bands shown on page 124.

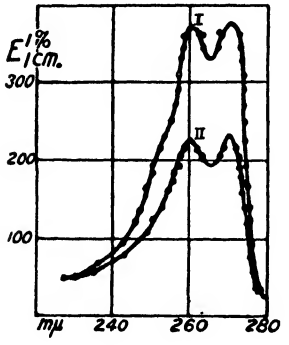


FIG 48

Reduction products of vitamin K_1 (curve I) and K_2 (curve II) These compounds still possessed a quinonoid structure.

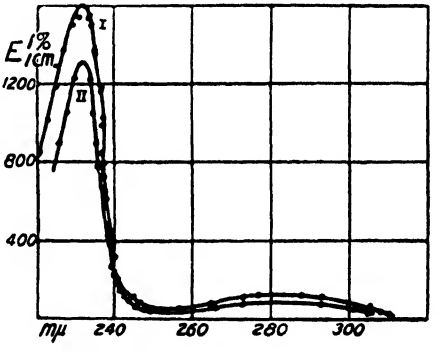


FIG. 49

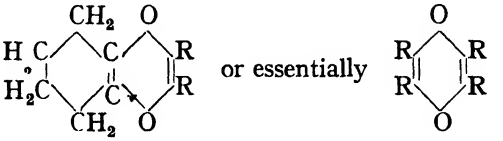
Diacetates of dihydro vitamins K_1 (curve I) and K_2 (curve II).

After Ewing *et al.*

Webb (see Fieser *et al.*, *J. Amer. Chem. Soc.*, 1939, **61**, 1927) and Ewing, Vandenbelt and Kamm (*J. Biol. Chem.*, 1939, **131**, 352), in extending the work of several groups of investigators, recorded for the K vitamins a group of four maxima in the region 240–280mμ, as well as a weaker band near 330mμ. Still weaker absorption at 400–450mμ, log $\epsilon < 2$, is to be expected. Ewing *et al.* examined the substances obtained by catalytic reduction of the vitamins, the non-quinonoid ring only being affected.

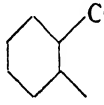
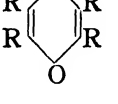
Reduced vitamin K_1	Reduced vitamin K_2
$(C_{31}H_{50}O_2)$.	$(C_{40}H_{60}O_2)$.
$\lambda_{max.}$ A, *ca* 2600, 2700	2600, 2700
log $\epsilon_{max.}$ 4·23	4·12

The absorptive part of both substances is



α-Tocopherylquinone, obtained by oxidation of α-tocopherol by means of ferric chloride, contains the same chromophoric grouping and shows $\lambda\lambda_{max.}$ 2625 and 2725 A, log ϵ 4·23 (Karrer and Geiger, *Helv. chim. Acta*, 1940, **23**, 455).

The substituted 1 : 4-naphthoquinones (Table I) thus exhibit spectra little different from the simple summation $a + b$:

(*a*)	$\lambda_{max.}$ A.	log $\epsilon_{max.}$	(*b*)	λ ϵ_{max} A.	log $\epsilon_{max.}$
	2430	4·02		*ca* 2600	*ca* 4·2
	2495	4·025		2706	
	3290	3·63		~ 4000–4500	*ca* 2·0

The above explanation is due to Morton and Earlam (*J. Chem. Soc.*, 1941, 159).

It is important to prevent access of light to dilute solutions of either K_1 or K_2 as both substances deteriorate unless they are kept in darkness.

The diacetates of dihydrovitamins K_1 and K_2 show $\lambda_{max.}$ 230mμ, $E^{1\%}_{1cm.}$ 1600 and 1300 respectively, and $\lambda_{max.}$ 285mμ, $E^{1\%}_{1cm.}$ *ca* 100. The diacetates are stable and highly potent, and Ewing *et al.* (*loc. cit.*) state that " it would seem that these compounds have the proper qualifications as reference standards for the vitamins ".

Doisy's synthetic vitamin K_1 shows :

$\lambda_{max.}$	243	249	260	269	325mμ
$E^{1\%}_{1cm.}$	410	425	395	395	75
$\epsilon_{max.}$	18,450	19,125	17,775	17,775	2,375

It seems likely that the synthetic dialkyl naphthoquinones may have definite therapeutic value. Thus Rhoads (*Surgery*, 1939, **5**, 794), in a careful review, records successes in dealing with the haemorrhage tendency in obstructive jaundice. " It is generally (put down to) plasma prothrombin deficiency, due in turn to avitaminosis K, resulting in failure of absorption of the vitamin from the intestine, a failure due probably to absence of bile salts." " As vitamin K is inexpensive it will probably be used routinely (in conjunction with bile salts) in patients with obstructive

jaundice before and after operation even though no prothrombin deficiency is found. The need for transfusions may be reduced."

The St. Louis school led by Doisy (*J. Biol. Chem.*, 1939, **131**, 327) describe in detail the technique of preparing vitamin K_2 by allowing moist fish meal to undergo bacterial putrefaction. A large number of bacteria, including B. coli, are effective. After 15 days at 32–40° C., with daily addition of water at 40°, the meal is dried and extracted with petrol ether. Subsequent putrefaction of this meal gave extracts 4–5 times more potent than those from the first putrefactive treatment and five or six successive putrefactions proved worth while. Yields of 35–50 units per gram of fish meal were obtained (vitamin K_2 1·5μg = 1 unit), or 1 unit in 150–1500μg of extracted solids. The anti-haemorrhagic compound is apparently also synthesised by bacteria in the lower portions of the intestinal tract. A most interesting problem presents itself in the search for possible precursors to vitamin K_2 in bacterial nutrition.*

* Note added in proof. The author has summarised recent work on Vitamin K in the *Annual Review of Biochemistry*, vol. 12 (1942).

CHAPTER VI

VITAMIN C AND VITAMIN P

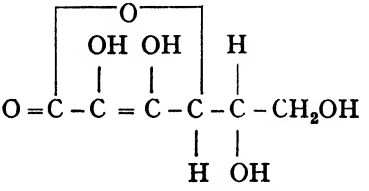
Tentative approaches towards the concept of deficiency diseases can be seen in quite early medical literature, and the history of scurvy is of particular interest from this point of view. That mariners deprived of fresh foodstuffs during long voyages were susceptible to scurvy was common knowledge, and as early as 1720 Kramer had placed it on record that the disease could be prevented by a well-chosen diet. The reputation of orange juice, lemon juice and lime juice as anti-scorbutics was mostly well deserved, although adulteration of the latter with orange juice may have been responsible for much of its efficacy. The scientific study of scurvy was placed on a sound basis by Lind in 1757. In 1883, Barlow made it clear that infantile scurvy was due to faulty diet, but it was not until the present century, and particularly after 1912, when the vitamin concept was " in the air ", that rapid progress began to be made.

Holst and Frohlich (*J. Hyg.*, 1907, **7**, 634) found that the guinea pig could be used as an experimental animal for the study of scurvy. Their choice was fortunate, as will be seen later. Progress in devising scorbutic diets and in biological technique was made by Cohen and Mendel (*J. Biol. Chem.*, 1918, **35**, 425) and LaMer, Campbell and Sherman (*J. Amer. Chem. Soc.*, 1922, **44**, 165) devised a method for the quantitative determination of the vitamin. Mellanby, Zilva and others, developed the biological groundwork for chemical studies. Considerable progress was made in the direction of concentrating the active principle from lemon juice, but great difficulties were encountered, owing mainly to the sensitivity of the vitamin to oxidation (Zilva, *Biochem. J.*, 1929, **23**, 1199 ; Bezssonoff, *Bull. Soc. Chim. biol.*, 1929, **11**, 294). Smith, Svirbely and King (*J. Biol. Chem.*, 1931, **94**, 491, 483) isolated an active crystalline substance from lemon juice after great labour.

In 1928, Szent Györgyi had isolated a hexuronic lactone $C_6H_8O_6$ from adrenal glands. He was actually studying tissue respiration and at that stage no connexion with vitamin C was apparent. His material was a crystalline, water-soluble substance, labile to heat, especially in the presence of oxygen, but its most obvious property was a powerful reducing

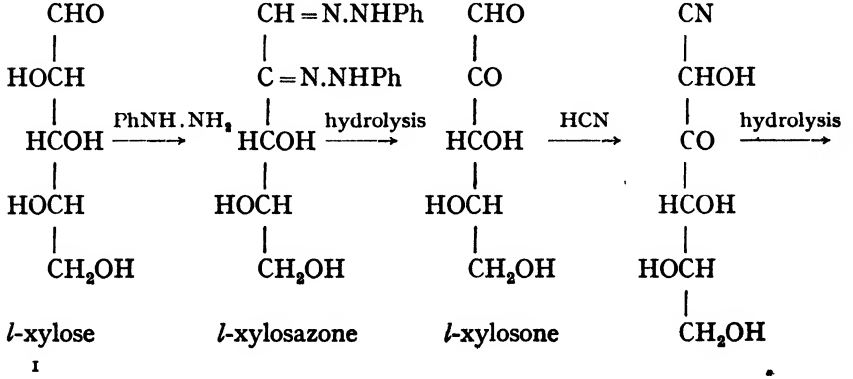
action. Tillmans and Hirsch (*Biochem. Z.*, 1932, **250**, 312) suggested that Szent Györgyi's " hexuronic acid " and the antiscorbutic vitamin were identical and this was soon confirmed (Waugh and King, *J. Biol. Chem.*, 1932, **97**, 325 ; Svirbely and Szent Györgyi, *Biochem. J.*, 1932, **26**, 865). It is interesting that the vitamin had actually been isolated some four years before the direct attack permitted the fact to be recognised.

The next step was to establish a structural formula. Much of the work was carried out on a very small scale using material prepared in Szent Györgyi's laboratory from paprika and adrenal glands. Haworth and Hirst and their collaborators discovered the essential facts very quickly (*J. Soc. Chem. Ind.*, 1933, **52**, 221, 481), and valuable contributions were made by Karrer *et al.* (*Helv. Chim. Acta*, 1933, **16**, 1161) and Micheel and Kraft (*Z. physiol. Chem.*, 1933, **222**, 235). The finer points in the structure were being elucidated when Reichstein, Grüssner and Oppenhauer (*Helv. Chim. Acta*, 1933, **16**, 1019) synthesised *d*-ascorbic acid and later *l*-ascorbic acid. This name, suggested by the Birmingham School, replaced " hexuronic acid ", although the American Medical Association recommended " cevitamic acid ". The substance is actually a new type of hexuronic lactone :



(See Haworth, Hirst *et al.*, *J.C.S.*, 1933, 1270, 1564 ; 1934, 62, 1556)

A number of synthetical procedures are now available for the preparation of vitamin C, some being patented. A typical synthesis is shown below :



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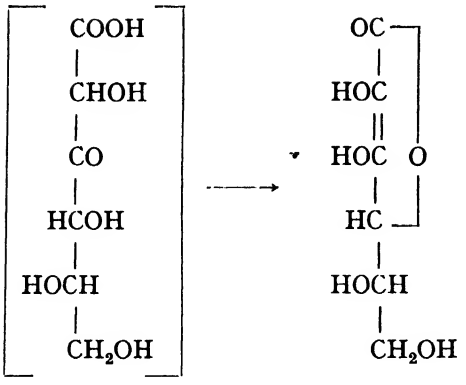


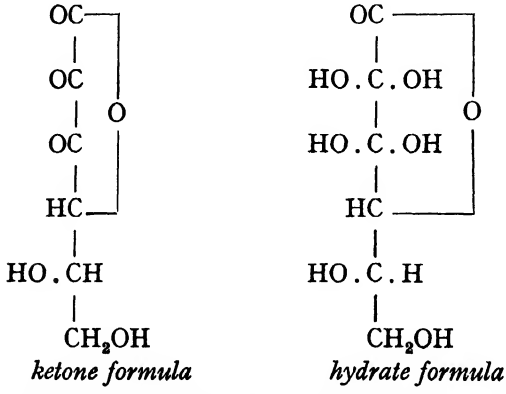
(intermediate)
3 Ketogulonic acid

Vitamin C.
(ascorbic acid)
(cevitamic acid in U.S. literature)

A large number of substances structurally akin to ascorbic acid are now known, and Zilva (*Biochem. J.*, 1935, **29**, 1612) has summarised the results of bio-assays carried out on such synthetic products. The most active was *l*-rhamno-ascorbic acid (1/5 the activity of *l*-ascorbic acid); *d*-ascorbic acid is biologically inactive.

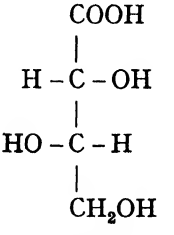
Ascorbic acid owes its acidity to dissociation of an enolic hydrogen and not to opening of the lactone ring. In aqueous solutions of $pH < 7{\cdot}6$ oxidation occurs in the presence of minute quantities of Cu^{++}, but the vitamin does not normally undergo autoxidation in plant and animal tissues. Ascorbic acid is reversibly oxidised by methylene blue, indophenols, and by a number of inorganic reagents. The product, dehydro-

dehydro-ascorbic



ketone formula *hydrate formula*

threonic acid

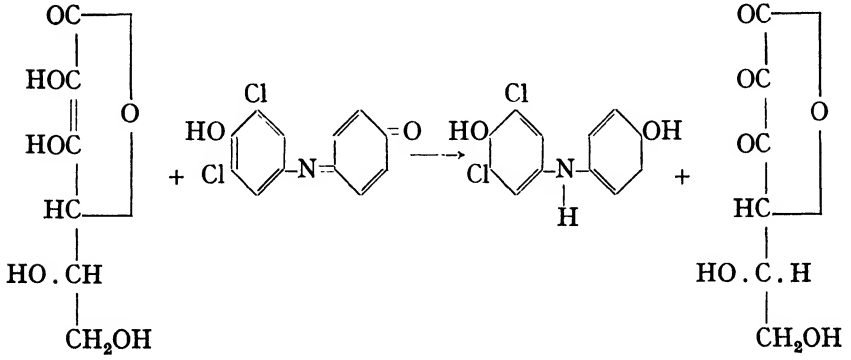


(See *Nature*, 1932, **129**, 576; **130**, 888)

ascorbic acid, is converted (irreversibly) at p*H* values >4 to another reducing substance, which in turn gives rise to oxalic acid and *l*-threonic acid.

Ascorbic acid is readily reduced by cysteine, glutathione and H_2S.

Bessey (*J. Amer. Med. Ass.*, 1938, **111**, 1290) reviews the methods of assay for vitamin C and its distribution in foodstuffs. Tillmans *et al.* (*Z.f. Untersuch. Lebensmitt.*, 1932, **63**, 1) established a parallelism between vitamin C in extracts from fruits and vegetables and the results of titrations with 2–6 dichloro-indophenol.



Although the test is not quite specific it is of proved value (King, *Physiol. Rev.*, 1936, **16**, 238; Harris, *Ann. Rev. Biochemistry*, 1934, **3**, 264; Emmerie and Eekelen, *Biochem. J.*, 1936, **30**, 25). Difficulties are met in special instances with reducing substances other than vitamin C, and care must be taken lest the ascorbic acid should undergo oxidation prior to the actual titration. The review by Bessey (*loc. cit.*) contains much valuable information which need not concern us in this work. In particular the procedures for applying the indophenol titration to urine and blood are discussed. One fact is of over-riding importance in connexion with vitamin C, *i.e.* that in the presence of air, the merest trace of Cu^{++} leads to almost complete destruction in a very short time. Cooking utensils containing copper even in minute proportions, are therefore to be avoided as containers for milk, or for cooking vegetables.

Vitamin C occurs in rapidly growing seeds like green peas, but not in dried seeds. It frequently, but not necessarily, accompanies carotenoids in vegetable products and there is little doubt that it stimulates growth in plants. Most animals appear to be able to synthesise ascorbic acid, the exceptions being the primates and guinea pigs. It was fortunate that Holst and Frohlich (*loc. cit.*) discovered the usefulness of guinea pigs so early. Human beings and guinea pigs are specially subject to scurvy during pregnancy and lactation. As far as species which do not need preformed vitamin C are concerned, there is no real evidence concerning the nature of the substance from which ascorbic acid is synthesised *in vivo*. It is interesting that human milk contains 4–5 times as much vitamin C as cows' milk; the calf can synthesise ascorbic acid in its own tissues

whilst the infant cannot do so. The distribution of vitamin C in the body shows that the highest concentrations occur in those tissues which have a high metabolic activity.

If dietary vitamin C is withheld, urinary excretion continues until the body reserves are depleted. When vitamin C is supplied again, the tissue reserves tend to be replenished before excretion *via* the urine reaches the normal value. These facts afford a basis (conveniently attainable by means of the indophenol titration) for analytical control of malnutrition in respect of the anti-scorbutic vitamin (*cf.* Harris and Ray, *Lancet*, 1935, **1**, 71). There has been much study of the functions of ascorbic acid, and it is quite clear that it plays an important part in calcium metabolism, but the mechanism of the process is unknown. It seems certain that ascorbic acid plays a part in hydrogen transport in enzyme systems, but the evidence has not yet been sifted very satisfactorily. An increased fragility in the blood vessels was at one time thought to be a symptom of vitamin C deficiency but is now regarded as due to shortage of vitamin P (*q.v.*).

The doses of vitamin C which are needed, are on the large side judged by the requirements in respect of fat-soluble vitamins:

average adult, vitamin C need	*ca* 25mg./day
infants " " "	*ca* 50mg./day
children 1–5 " " "	up to 150mg./day
pregnant and lactating women " " "	150mg./day

There are indications that quite large doses may be prescribed in certain acute diseases, but there are very serious gaps in knowledge concerning the therapeutic use of ascorbic acid.

The first systematic spectrophotometric work on ascorbic acid was carried out by Hirst and Herbert (*Nature*, 1932, **129**, 205, *J. Chem. Soc.*, 1933, 1270; Baird *et al.*, *ibid.*, 1934, 63). It is essential to use pure (Cu^{++}-free) water, and to avoid oxidation or excessive exposure to u.v. rays if accurate results are to be obtained for solutions in pure water ($c = 0{\cdot}002\%$); $\lambda_{max.}$ occurs at 265mμ, log $\epsilon_{max.}$ 3·97. In the presence of a little mineral acid or in alcoholic solution $\lambda_{max.}$ occurs at 245mμ, the ϵ value being practically unchanged. The extinction coefficient is notably high in comparison with the ϵ values for the following substances:

	$\lambda_{max.}$ mμ	$\epsilon_{max.}$	solvent
Acetylpyruvic acid	285	4,000	water
Dihydroxymaleic acid	290	6,700	water
Laevulic acid	263	24	water
Glucosone	310	13	alkali
Acetone	265	15	alcohol

Thin layers of more concentrated solutions of ascorbic acid show λ_{max} 240–245mμ. Departures from Beer's Law are thus partially due to the drift in $\lambda_{max.}$ from 265 to 245mμ with increasing concentration. In dioxan, the maximum occurs at 245mμ and in alkali at 265mμ, $\epsilon_{max.}$ being approximately constant throughout.

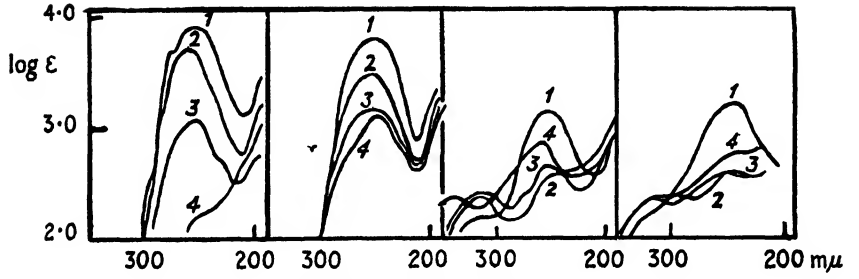
Mohler (*Helv. chim. Acta*, 1938, **21**, 485) has carried out a careful study of the effect of copper ions in catalysing the oxidation of ascorbic acid. By adding small quantities of potassium cyanide this effect can be eliminated. Fig. 50 illustrates the results of the investigation clearly.

Spectrophotometric methods for the determination of vitamin C in biological fluids have been used by Robertson (*J. Soc. Chem. Ind.*, 1934, **53**, 277); Johnson (*Biochem. J.*, 1936, **30**, 1430) and Chevallier and Choron (*Bull. Soc. Chim. Biol.*, 1937, **19**, 523). The eye fluids, aqueous and vitreous humour as well as the lens, contain ascorbic acid. Johnson's method depends upon taking two portions of humour each consisting of exactly 1 c.c. The first is at once acidified with 0·2N.HCl and diluted with freshly distilled water free from Cu^{++}, so as to give a solution of humour in 0·02N HCl. The spectrum is then photographed. The second portion is diluted with water to 80% of the final volume and sufficient copper sulphate solution is added to give a final Cu^{++} concentration of 2 mg./1l. Oxidation at once sets in, and an hour later all the ascorbic acid has been converted to dehydro-ascorbic acid, the absorption of which is negligible. The spectrum of the solution is then obtained. The absorption shown by the second solution represents the contribution made to the total by constituents of the humour other than the vitamin C. Subtraction of the two curves gives the nett absorption due to ascorbic acid, and the concentration can be calculated from the absorption observed with the solutions containing a known amount of the pure acid.

VITAMIN P

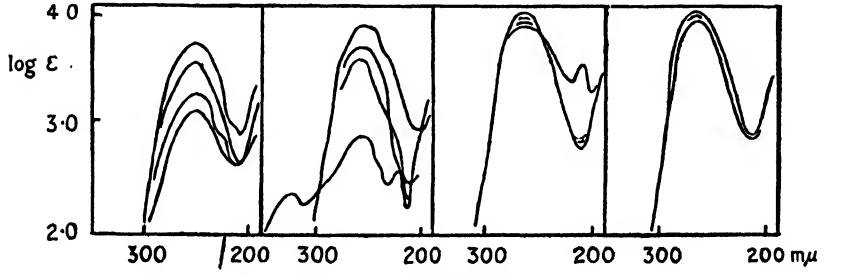
Szent Györgyi and his colleagues claim to have established the existence of a vitamin P capable of controlling haemorrhage in a number of clinical states. Trials on guinea pigs indicate that the vitamin is capable of restoring to their normal state capillaries which have become fragile and permeable. The vitamin occurs in lemon juice and red peppers, and a crystalline material *citrin* was isolated from the former (*Nature*, 1936, **138**, 27; 1937, **139**, 326; *cf.* also Zilva, *Biochem. J.*, 1937, **31**, 915). In certain human conditions, injections of citrin are thought to improve the permeability of the capillaries.

Szent Györgyi (*Z. physicl. Chem.*, 1938, **255**, 126) obtained citrin as small yellow needles, $C_{28}H_{36-38}O_{17}$, insoluble in ether, chloroform and



Concentration $CuSO_4$ — *l*-ascorbic acid in water.

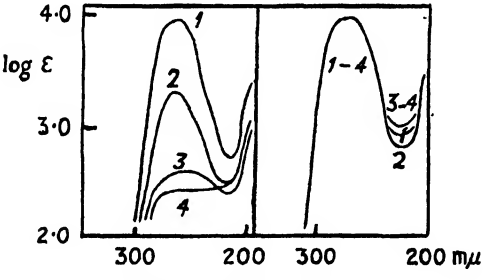
	M/2,000	M/2,000	M/2,000	M/1,000
	0	M/200,000	M/20,000	M/1,000
Air bubbled through	1 fresh	1	1	fresh
	2 1 hr.	2	1½	hrs.
	3 for 5 hrs. (3)	3	5	hrs.
	4 3 hrs. (4)	4	24	hrs.



l-ascorbic acid M/2,000
$CuSO_4$ M/200,000

KCN M/200,000 M/20,000 M/2,000 M/1,322

1 fresh
2 after 1½ hrs.
3 after 5 hrs.
4 after 24 hrs.



M/2000 ascorbic acid.
Buffered at p*H* 5·29

KCN 0 M/2,000

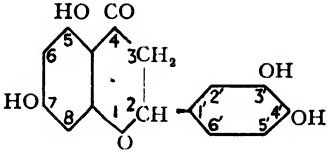
1 fresh
2 after 1 hr.
3 after 5 hrs.
4 after 24 hrs.

FIG. 50

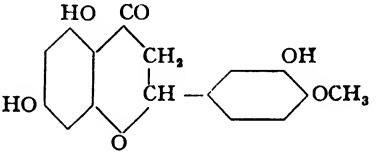
After Mohler and Lohr (*Helv. chem. Acta*, 1938, **21**, 485)

acetone, slightly soluble in water or alcohol and readily soluble in pyridine. With alkali it yields colourless solutions which soon turn yellow. It is labile towards oxidising agents, and on boiling in acid solution gives a strongly reducing hydrolysate. It appears to function as an intermediary between polyphenoloxidase and oxygen or peroxidase —peroxide-ascorbic systems. The molecule contains one methoxyl group.

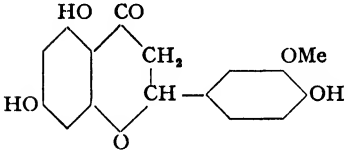
Citrin is however heterogeneous, containing hesperidin and eriodictyol glucoside.



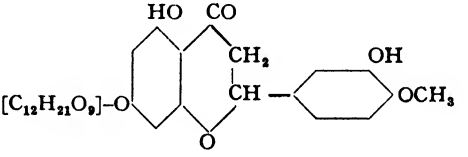
eriodictyol
5,7,3′,4′-tetrahydroxyflavanone
(occurs as glucoside)



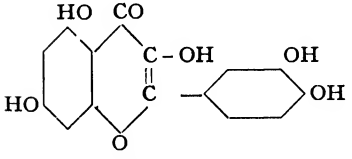
hesperetin
5,7,3′trihydroxy,4′-methoxy-flavanone



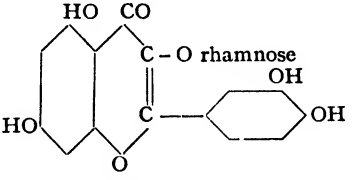
homoeriodictyol



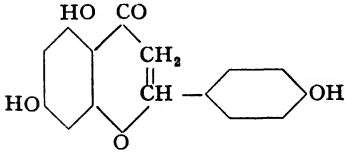
hesperidin
(hesperetin rhamnoglucoside)



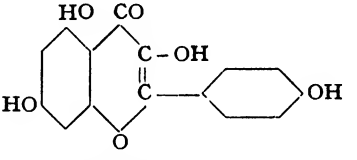
quercitin



quercitrin



naringenin



kaempferol

Scarborough (*Biochem. J.*, **33**, 1400) has examined critically the work of Szent Györgyi's group. He tested (*a*) a crude powder free from ascorbic acid, deposited in the desiccation of orange juice; (*b*) a crystalline material, *m.p.* 255–256° (Glaxo Laboratories), obtained by recrystallisation from pyridine; (*c*) citrin (Roche, Glaxo), a mixture of flavanones from orange peel. Scarborough concluded that his tests confirmed the presence in orange and lemon juices of a substance of flavanone nature, which could increase the resistance of capillary walls to the application of pressure.

Whether vitamin P is actually hesperidin or eriodictyol glucoside or a contaminant is not yet certain. The preparations are, however, effective enough whether administered orally, intramuscularly or through the rectum. Some critics had doubted the validity of the vitamin P concept, but although Scarborough found it necessary to be cautious in interpreting clinical experience, it seems that a good case for vitamin P has been made out.

Wilson (*J. Amer. Chem. Soc.*, 1939, **61**, 2303) observed that lemon juice dried in the presence of boric acid produced a brilliant yellow colour. Measurable colour was obtained with 0·5 c.c. of solution. Hesperetin and naringenin and other flavanones gave a negative reaction whereas quercitrin and kaempferol gave positive results. This test appears to afford a distinction between flavanones and flavonols which may be useful (for details concerning the preparation of the borocitric reagent, etc., see the original paper).

Lajos and Gerendás (*Biochem. Z.*, 1937, **291**, 229) have studied the absorption spectra of citrin and other flavone-like substances. The results are summarised in Table VII and it is clear that quercitrin, which is a flavonol derivative, shows a spectrum differing considerably from that characterising the flavanone glycosides. It would be interesting to discuss these spectra from the point of view of chromophoric groups but too great a departure from the subject matter would be entailed (*cf.* Morton and Sawires, *Jour. Chem. Soc.*, 1940, 1052).

The following method for the detection of hesperidin and eriodictyol glycoside is given with reserve :—

(*a*) extract the product thoroughly with cold amyl alcohol.

(*b*) wash the amyl alcohol extract with 1% HCl to remove anthocyanins.

(*c*) transfer to ethyl alcohol and purify through the lead salt (*Z. physiol. Chem.*, 1938, **255**, 126).

(*d*) decompose the lead salt, suspended in alcohol, by means of sulphuric acid.

(*e*) determine the absorption spectrum of the alcoholic solution; if "citrin" occurs two bands will be shown, 320–330mμ and 280–290mμ.

(*f*) determine the spectrum in N/40 NaOH; a new band near 360mμ will appear if hesperidin is present, but if eriodictyol glycoside is present the absorption will show maxima at 263 and 320mμ. If both are present the results will be confusing and it will be necessary to attempt the difficult task of separating the two glycosides or else to fall back upon empirical methods.

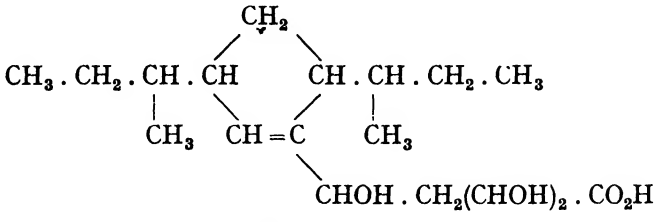
(The above arises out of work in progress in which the writer has assisted Dr. A. Pollard, and it seems unlikely that hesperidin will turn out to be vitamin P.)

TABLE VII

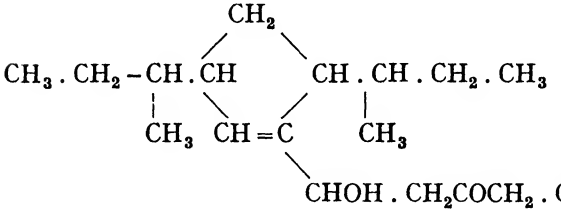
Substance	Solution	λ_{max} mμ	log $E^{1\%}_{1cm.}$	$\lambda_{min.}$ mμ	log $E^{1\%}_{1cm.}$
citrin	M/10,000 in alcohol (or water)	284	2·08	248	1·70
		330	1·80	310	1·70
hesperidin	M/10,000 in alc. (0·0646%) -	278	2·02	255	1·79
		324	1·56	315	1·35
eriodictyol	M/10,000 in alc. (0·0333%)	290	2·54	251	1·72
		326	2·16		
homo-eriodictyol	M/10,000 in alc. (0·0302%) -	290	2·26	260	1·66
		328	2·33	303	2·14
citrin	in N/40NaOH (after 2 hours)	285	2·27	265	2·14
		360	2·12	320	1·91
hesperidin	ditto	287	2·32	265	2·01
		360	2·02	340	1·93
eriodictyol	ditto	263	2·61	242	2·54
		320	2·64	290	2·50
citrin	in N.NaOH (after 2 hours)	279	2·30	258	2·14
		380	2·06	340	2·04
hesperidin	ditto	279	2·31	262	2·14
		373	2·26	330	2·09
citrin	HCl (hydrolysis?)	285	2·05	249	1·69
		420	1·74	355	1·51
quercitrin	M/10,000 in alc. (0·034%) -	258	2·75	240	2·55
		375	2·75	300	2·40

Plant Hormones

Kögl's important work (*cf. Ber.*, 1935, **68**, 16) on the auxins led to the following structures:



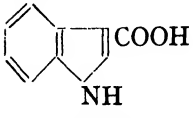
auxin-*a*.



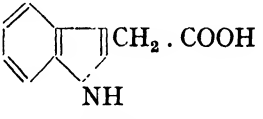
auxin-*b*.

The spectra of these substances do not appear to have been recorded. Auxin-*a*, from its structure, might be expected to show continuous end absorption at wave-lengths $< 240m\mu$. Auxin-*b*, as a derivative of aceto-acetic acid, is likely to show more interesting absorption. The carbonyl absorption of low intensity at *ca* $280m\mu$ might be displaced to *ca* $300m\mu$ with ϵ_{max} of the order 200. Since the substance is obviously potentially enolisable, the spectra in different solvents and in excess of alkali should exhibit considerably different intensities (*cf. J.C.S.*, 1934, 883).

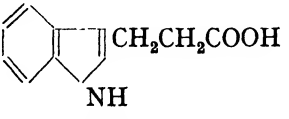
Heteroauxin or indole-3-acetic acid exhibits the characteristic selective absorption of indoles. The utility of the absorption spectrum is, however, greatly diminished by the fact that the biological response is much more specific:



inactive



very active



inactive (Kogl)

$\lambda_{max.}m\mu$	280	225·5	281	226
log $\epsilon_{max.}$	3·8	4·46	4·05	4·56

(Grinbaum and Marchlewski, *Bull. Intern. Acad. Polon. Sci., Classe, Sci. Math. Nat.*, 1937 A, 171.)

A similar situation arises with the naphthalene-acetic acids, which show the characteristic spectrum of the naphthalene nucleus, but there is no specific correlation between root formation and selective absorption.

CHAPTER VII

THE VITAMIN B COMPLEX

The literature of vitamin studies grows at a great pace, and it is necessary to take stock of the position very frequently. As biological experimentation is extended to more and more species, new and apparently specific reactions to different basal diets are recorded, additional vitamins are postulated and the mere problem of nomenclature becomes formidable. Basic concepts like those of a hormone, a vitamin, an enzyme, and an essential amino-acid lose something in precision as complex interrelationships are unveiled. Bacterial- and insect-nutrition are subject to the need for essential organic substances which cannot be synthesised by the growing organism, and quite unexpected effects are frequently observed. For example, the urine of patients suffering from pernicious anaemia lacks a mosquito growth factor which is present in normal urine. The attack is thus being made on an ever-widening front and the data are increasingly complex, but there is also a greater opportunity for insight into the underlying order. Numerous instances could be quoted of apparently unrelated investigations which conjointly provide highly significant clues for the study of problems other than the immediate objectives. It seems certain that one outcome of the comparative approach to the study of accessory food factors will be an interesting sidelight on evolution. The puzzling fact, for example, that the sterols in ergot, in yeast and in the garden worm are extremely rich in ergosterol whilst the cholesterol of higher forms such as mammals and man has only traces of a distinct but related provitamin D, may find an explanation. In the course of the evolutionary process primitive needs may be met by increasing powers of synthesis but new functions may make further " vitamin " demands on the diet, or new mechanisms for the utilisation of essential substances may be evolved.

In surveying the field of the water-soluble vitamins included in the vitamin B complex it is only possible to present a " snapshot " picture. On every hand order is being brought out of confusion, but we can hope for a much more tidy and exact picture in the future. For the purposes of this book, discussion will be facilitated by defining the terms as they are now used :

	Synonyms	*Characteristics*
Vitamin B_1	aneurin thiamin (in U.S.A.)	Anti-beri-beri in man, anti-polyneuritis in experimental animals.
Vitamin B_2	riboflavin *G* and *lactoflavin* (both obsolete) 6, 7-dimethyl-9 (1′ ribityl) *iso* alloxazine	A component of the oxidation-reduction system of cells, necessary for normal growth in chicks and for the prevention of cataract in experimental rats.
P–P factor	nicotinamide	Prevents human pellagra and canine black-tongue.
Vitamin B_4	B_8	A factor associated with specific symptoms of paralysis in rats and chicks.
Vitamin B_5		A factor needed for maintaining weight in the pigeon.
Vitamin B_6	(or pyridoxin adermin)	The anti-dermatitis factor for chicks, resembles, but is not necessarily the same as H, Y, I and W.
Filtrate factor	includes nicotinamide but may be complex.	Effective in human pellagra, canine black-tongue, nutritional dermatosis of chicks.

Vitamin B_1

There is a special interest in vitamin B_1 because of its importance in the history of the vitamin concept and of the extremely difficult task its isolation presented to chemists. The work of Dutch physicians, notably Ejkman and Grijns, in the East Indies, established the nutritional origin of beri-beri and the possibility of producing a similar condition experi-

mentally in fowls. It is now known that even the richest food sources contain only 20–30 parts per million of the vitamin, and, in retrospect, it is not surprising that 25 years of labour were needed before the pure substance could be obtained in sufficient quantity for determining its constitution.

Crystalline specimens of the vitamin (prepared by Jansen *et al.*, *Rec. trav. chim.*, 1933, **52**, 366; Van Veen, *Z. physiol. Chem.*, 1932, **208**, 125; Ohdake, *J. Agric. Soc. Japan*, 1931, **1**, 775; Windaus, Tschesche and Ruhkopf, *Nachr. Ges. Wiss. Göttingen*, 1932, **3**, 342; Kinnersley, O'Brien,

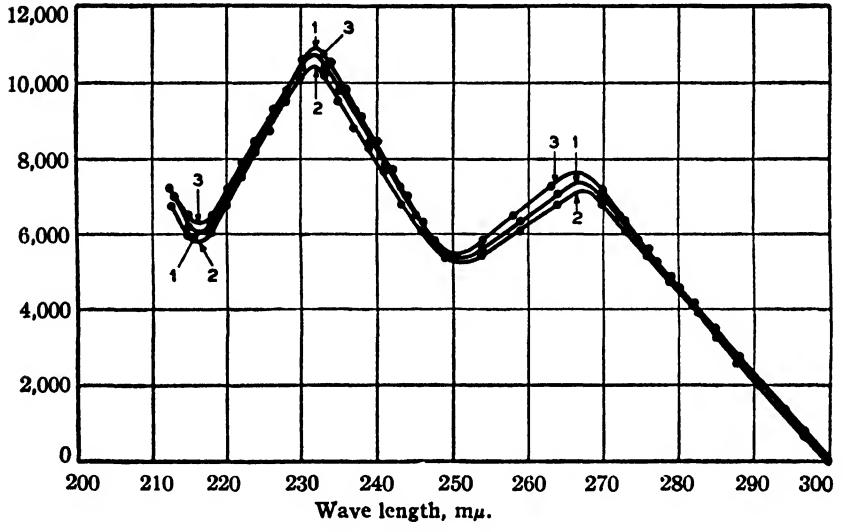


FIG. 51

Curve 1. Natural vitamin B_1 (hydrochloride).
2. Synthetic vitamin B_1 (hydrochloride).
3. ,, ,, (hydrobromide).

After Cline, Williams and Finkelstein

Peters and Reader, *Biochem. J.*, 1933, **27**, 225, 232; Peters and Philpot, *Proc. Roy. Soc.*, 1933, **113B**, 48) were almost identical, but difficulty was experienced in ascertaining the empirical formula. After repeated analyses of a number of highly purified specimens $C_{12}H_{16}O\ N_4S\ .\ 2HCl$ gained acceptance. It must be remembered that a yield of 62 mg. of this substance from 50 kg. of yeast (Peters and his colleagues) could only be obtained after many improvements in technique had been made.

A good deal of work had already been carried out on the absorption spectra of vitamin B_1 concentrates and on the crystalline vitamin. Heyroth and Loofbourow (*Bull. Basic Sci. Research*, 1931, **3**, 237) sus-

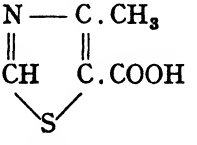
pected the presence of purine or pyrimidine-like substances but the material available only permitted very tentative conclusions to be drawn. Windaus, Tschesche and Grewe (*Z. physiol. Chem.*, 1934, **228**, 27) subjected crystalline vitamin B_1 to oxidation by means of nitric acid and obtained a crystalline degradation product, later shown (Clarke and Gurin, *J. Amer. Chem. Soc.*, 1935, **57**, 1876) to contain a thiazole nucleus. Kuhn, Rudy and Wagner-Jauregg (*Ber.*, 1933, **66**, 1950) had earlier isolated a fluorescent colouring matter *thiochrome* from yeast. Barger, Bergel and Todd (*Nature*, 1935, **136**, 259; *Ber.*, 1935, **68**, 2257) obtained this compound by oxidation of vitamin B_1, and Kuhn and Vetter (*Ber.*, 1935, **68**, 2384) published similar findings. Thiochrome was finally synthesised by Todd, Bergel and Jacob (*J. Chem. Soc.*, 1936, 1555).

R. R. Williams and his colleagues attacked the vitamin B_1 problem with admirable persistence, concentrating their efforts on isolating sufficient material for chemical study. One important advance was in the use of quinine for eluting the vitamin from the fuller's earth used as an adsorbent in an early stage of the process. By 1934, a few grams of the pure vitamin were available, and in less than a year the structure was approximately known. In Williams' own words: " In choosing an agent for splitting, it is obviously desirable to use one so gentle in its action that cleavage will occur only at the weakest point in the molecule. This sort of tool we found in sodium sulphite. Again, the important discovery was an accident. It arose from a previous attempt to preserve rice polish extracts against bacterial decay by the use of sulphites. The bacterial action had been stopped, but the vitamin had been destroyed promptly and completely at room temperature. This was turned to account by applying the action of sodium sulphite to the pure vitamin, from which we thus obtained two fragments of nearly equal size, quantitatively in the proportions in which they existed in the original."

$$C_{12}H_{16}N_4OS \cdot 2HCl \xrightarrow{Na_2SO_3} \underset{\text{I}}{C_6H_9N_3SO_3}\ (\text{aminosulphonic acid}) + \underset{\text{II}}{C_6H_9NSO} + 2NaCl$$

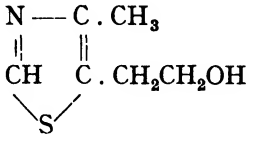
acidic basic

Fragment II undergoes oxidation with nitric acid to give a



compound first prepared in 1890, but identified in Williams' product by

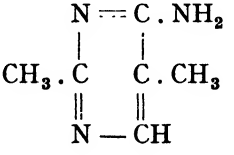
Clarke and Gurin (*loc. cit.*). Compound II contained an alcoholic hydroxyl replaceable by chlorine, and its structure was shown to be



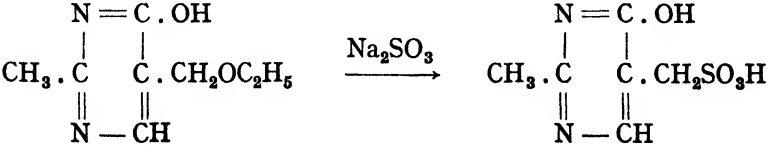
4-methyl-5-(β-hydroxyethyl)thiazole. Identification of the acidic fragment I proved difficult, but the following considerations illustrate the main steps:

I		III
$C_6H_9N_3SO_3$	$\xrightarrow[\text{drastic hydrolysis with acid}]{H_2O}$	$C_6H_8N_2SO_4 + NH_3$ oxy-sulphonic acid
I (or III)	$\xrightarrow[200°]{H_2O}$	sulphuric acid set free
I (or III)	$\xrightarrow[\text{alkali}]{}$	sulphurous acid set free
I	$\xrightarrow[\text{liquid ammonia}]{2Na \text{ in}}$	sodium sulphite + **IV**

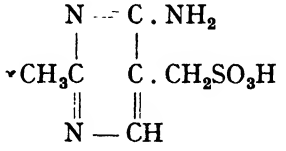
It was clear that **I** and **III** contained a sulphonic acid group. The absorption spectra and empirical formulae strongly suggested a pyrimidine nucleus. Compound **IV** is in fact 2,5-dimethyl-6-amino-pyrimidine,



but as no previous work had been published on such a substance a special investigation of synthetic 6-substituted pyrimidines was necessary to establish its constitution. Extension of this work showed that 2-methyl-5-ethoxymethyl-6-hydroxy-pyrimidine gave 2-methyl-6-hydroxy-pyrimidine 5-methylsulphonic acid

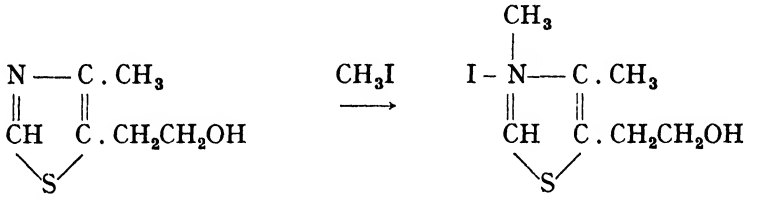


This proved to be identical with **III** so that the acidic cleavage product I has the structure

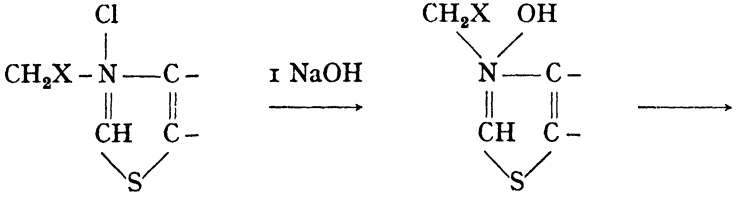


and it is obvious that the pyrimidine ring in vitamin B_1 must be attached to the rest of the molecule at position 5 where the SO_3H group has been introduced.

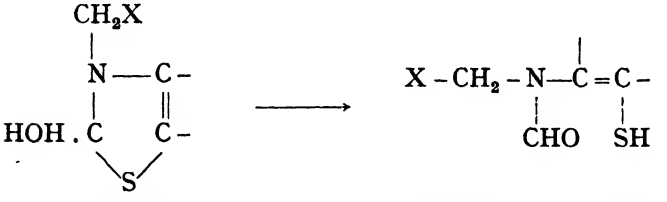
Now fragment **II** will add on a molecule of methyl iodide in the



expected way for a tertiary nitrogenous base. This compound behaves in an unusual way on titration with alkali. Vitamin B_1 chloride behaves similarly and this fixes the point of attachment of the thiazole ring. After the addition of one equivalent of alkali, further small additions cause an immediate increase in alkalinity followed by a slower decrease indicating a molecular rearrangement. The slow utilisation of excess alkali continues until three equivalents have been added. Subsequently the alkalinity rises steadily. The characteristic properties of quaternary thiazole salts are due to two rearrangements:

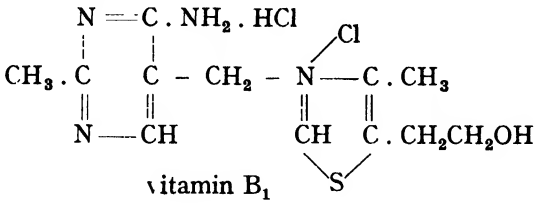


quaternary salt quaternary base



carbinol aldehyde-sulph-hydryl

the latter using the third molecule of sodium hydroxide. If X is the remainder (pyrimidine portion), the constitution of vitamin B_1 will be



vitamin B_1 — aneurin chloride hydrochloride

Application of absorption spectra to the structural problem

(*a*) Thiazole fragment

Ruehle (*J. Amer. Chem. Soc.*, 1935, **57**, 1887) showed that the basic fragment reacted with HCl:

$$C_6H_9NOS + HCl \longrightarrow C_6H_8NSCl + H_2O$$

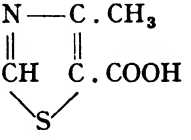
without appreciable change in absorption spectrum, indicating substitution of Cl for OH on a side chain. The probability that S formed part of a ring led to a comparison with substituted thiazoles.

	$\lambda_{max.}$ mμ	$\epsilon_{max.}$	λ_{min} mμ	$\epsilon_{min.}$
Basic cleavage product hydrochloride	252	4200	227	1350
4-methyl-thiazole-hydrochloride -	251	4000	220	550
2,4-dimethyl-thiazole-hydrochloride -	253	4500	220	50
2-hydroxy-4-methyl-thiazole-hydrochloride - - - - - -	248	4000	227	2500

Formation of a quaternary salt with methyl iodide led to a change in absorption:

	$\lambda_{max.}$ mμ	$\epsilon_{max.}$
Basic cleavage product methiodide - - -	227	12500
4-methyl thiazole ethiodide - - - -	227	13200

and the similarity noted above was very encouraging. When the compound



was synthesised and found to be spectroscopically equivalent to the oxidation product of Windaus (see p. 142) the structure of the thiazole fragment was established.

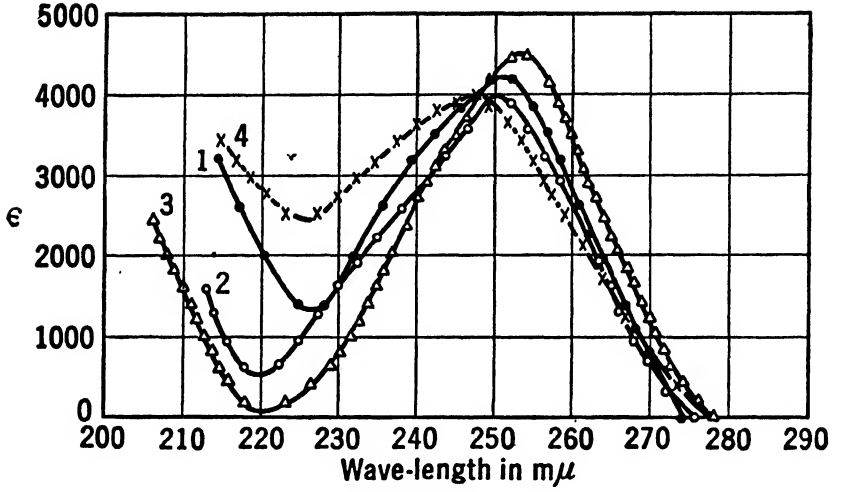


FIG. 52

1 ●——● Basic cleavage product hydrochloride.
2. o——o 4-methyl-thiazole-hydrochloride.
3 △——△ 2,4-dimethyl-thiazole-hydrochloride.
4. X——X 2-oxy-4-methyl-thiazole-hydrochloride.

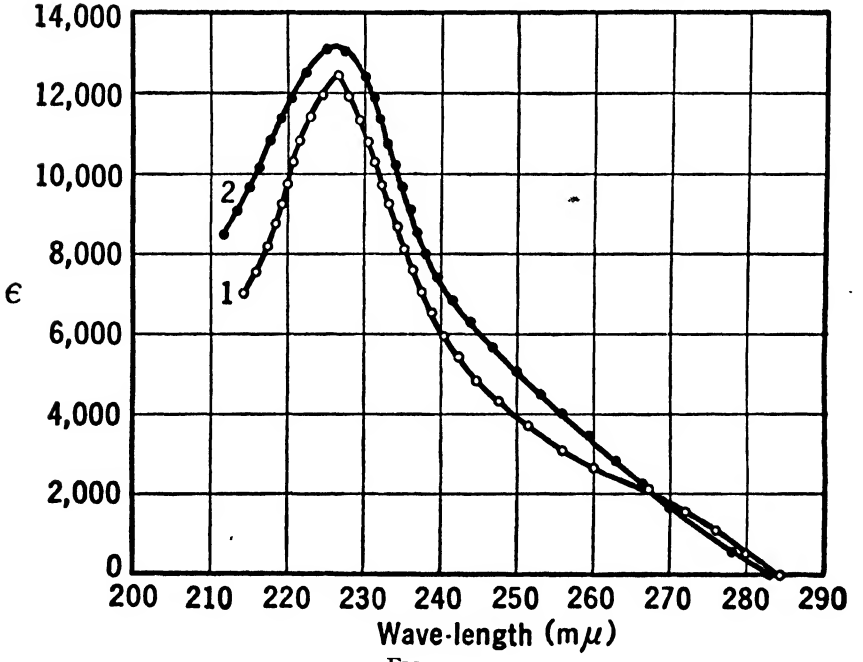


FIG. 53

1. o——o Basic cleavage product methiodide.
2. ●——● 4-methyl-thiazole-ethiodide.

After Ruehle

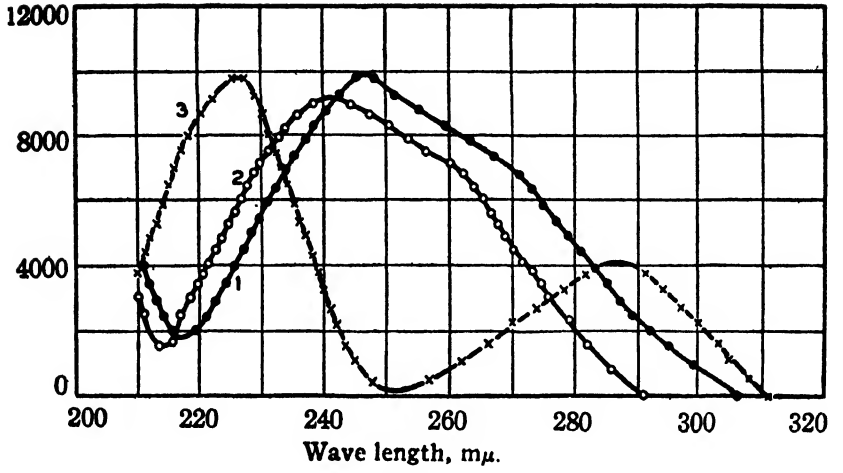


FIG. 54

1. Amino sulphonic acid (I).
2. 2,4-dimethyl-6-amino-pyrimidine.
3. 4,6-dimethyl-2-amino-pyrimidine.

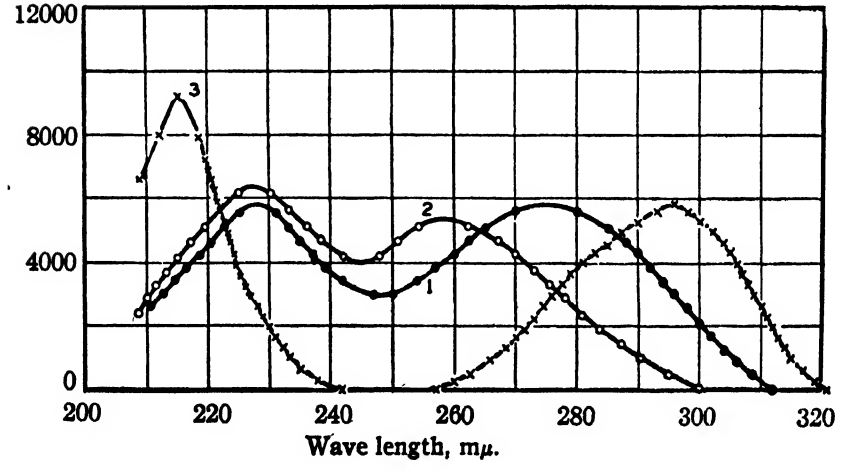


FIG. 55

1. Oxy-sulphonic acid (III).
2. 2,4-dimethyl-6-oxy-pyrimidine.
3. 4,6-dimethyl-2-oxy-pyrimidine.

After Williams, Buchman and Ruehle

(*b*) Acidic fragment

The splitting off of ammonia

$$\underset{\text{II}^{\nu}}{C_6H_9H_3SO_3} \xrightarrow[HCl]{H_2O} \underset{\text{IV}}{C_6H_8N_2SO_4} + NH_3$$

involves replacement of NH_2 by OH in a manner recalling 2-amino- and 6-amino-pyrimidines.

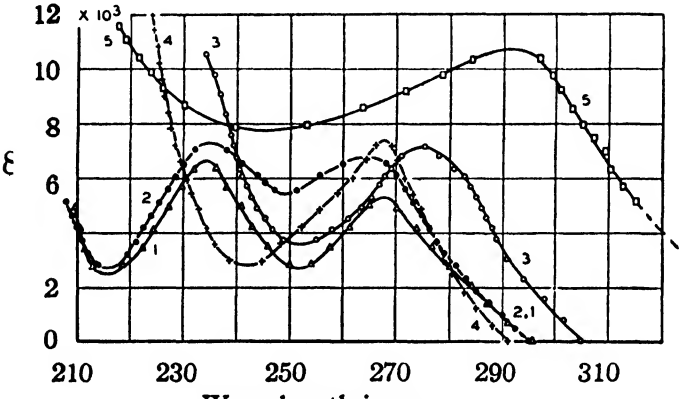


FIG. 56

1. Liquid ammonia cleavage product of vitamin.
2. 2,5-dimethyl-6-amino-pyrimidine.
3. 4,5-dimethyl-2,6-diamino-pyrimidine
4. 5-ethyl-4,6-diamino-pyrimidine.
5. 4-methyl-5,6-diamino-pyrimidine.

After Ruehle

Spectroscopic comparisons were therefore made :

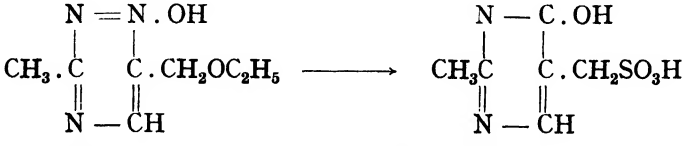
	$\lambda_{max.}$ mμ	$\epsilon_{max.}$	$\lambda_{min.}$ mμ	ϵ_{min}
amino-sulphonic acid - -	246	10000	218	1900
	~268	7000		
2,4-dimethyl-6-amino-pyrimidine	241	9100	214	1600
	~260	7200		
4,6-dimethyl-2-amino-pyrimidine	227	10000	252	100
	287	4100		
oxy-sulphonic acid - - -	229	5900	248	2800
	275	5800		
2,4-dimethyl-6-oxy-pyrimidine -	228	6200	245	4000
	258	5300		
4,6-dimethyl-2-oxy-pyrimidine -	215	9100	250	v. low
	296	5900		

The change in spectrum when NH_2 in the 6 position is replaced by OH is so strikingly reproduced that Ruehle could say: "We thus had proof of the presence of a pyrimidine ring and strong evidence for the location of one of the substituent groups months before the complete structure of this cleavage product was known and again could go ahead with confidence that other configurations were excluded." At this point six different isomerides were possible for the acidic cleavage product, even with an amino group established at position 6. Fortunately, the cleavage product $C_6H_{10}N_4$ (V) obtained from aneurin by treatment with liquid ammonia turned out to be a diamino compound, and it was possible to compare the spectrum of this substance with those of the three possible diamino-pyrimidines with one NH_2 at position 6.

	$\lambda_{max.}$ mμ	$\epsilon_{max.}$	$\lambda_{max.}$ mμ	$\epsilon_{max.}$
liquid ammonia cleavage product -	234	6500	215	2500
	268	5250	252	2800
2,5-dimethyl-6-amino-pyrimidine	235	7200	215	2800
	265	6700	249	5500
4,5-dimethyl-2,6-diamino-pyrimidine - - - - - -	275	6250	252	3600
5-ethyl-4,-6-diamino-pyrimidine -	268	7400	241	2900
4-methyl-5,6-diamino-pyrimidine	292	10800	247	6800
2-methyl-6-oxy-5-methylsulphonic acid - - - - - -	229	6000	210	3000
	277	4800	250	3000

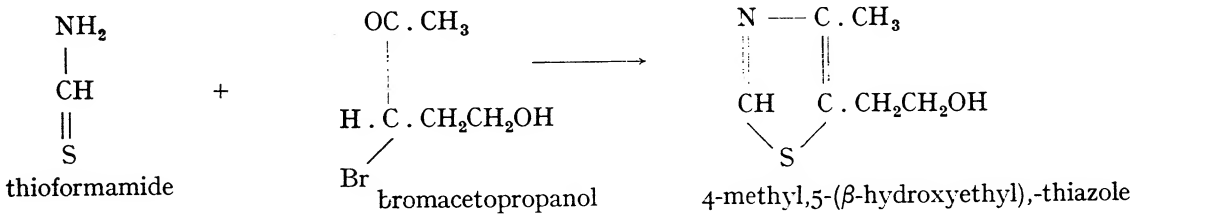
The resemblance between V and 2,5-dimethyl-6-amino-pyrimidine suggested a methylene bridge between the pyrimidine and thiazole portions in aneurin, and in fact treatment of the original acidic cleavage product with sodium in liquid ammonia gave 2,5-dimethyl-6-amino-pyrimidine.

The final difficulty lay in deciding which of the two methyls occurs at the point of attachment of thiazole to pyrimidine. The synthetic derivative:

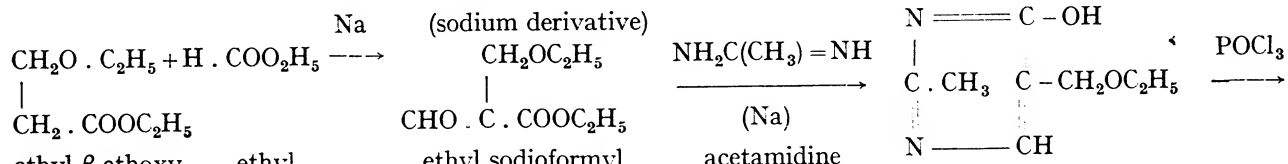


yields a sulphonic acid, but as the compound does not melt, strict proof of its identity with degradation product III is not easily obtained. Cline, Williams, Ruehle and Waterman (*J. Amer. Chem. Soc.*, 1937, **59**, 530) therefore used a spectroscopic criterion which may well prove widely applicable.

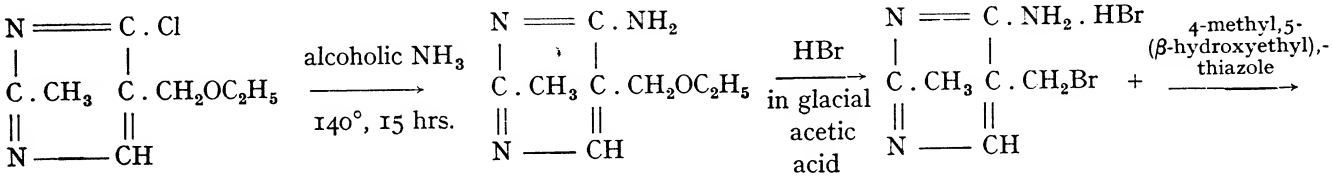
SYNTHESIS OF ANEURIN

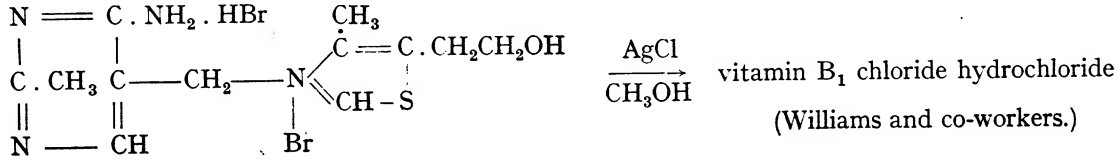


thioformamide — bromacetopropanol — 4-methyl,5-(β-hydroxyethyl),-thiazole



ethyl β-ethoxy propionate — ethyl formate — ethyl sodioformyl β-ethoxy propionate — acetamidine — 2-methyl 5-ethoxymethyl 6-oxypyrimidine



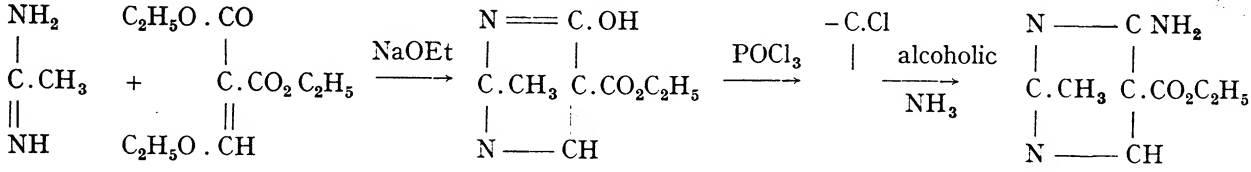


vitamin B_1 chloride hydrochloride

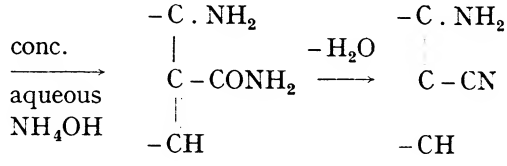
(Williams and co-workers.)

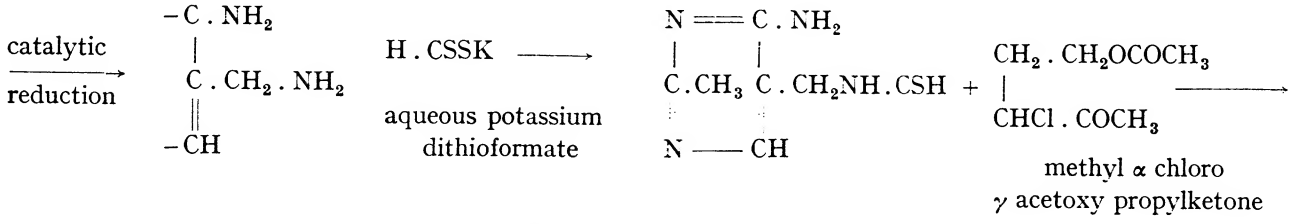
Turn through 180°.

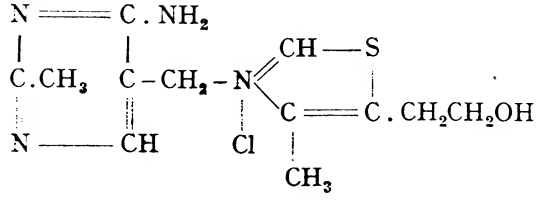
SYNTHESIS OF ANEURIN—*Continued*



Ethyl ethoxymethylene malonate





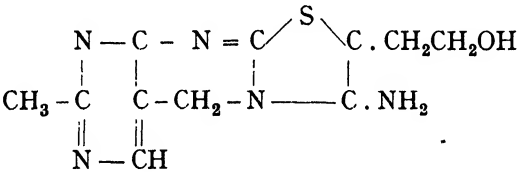


(Todd and Bergel.)

" The synthetic sulphonic acid was also compared with the natural with respect to ultra-violet absorption. Close agreement was observed. Since no melting point was available as a criterion of identity, and since experience with alkylated oxypyrimidines had indicated that ultra-violet absorption is not a definitive index of the position of alkyl substituents, it still appeared possible that the synthetic oxysulphonic acid was merely isomeric and not identical with the natural. This was especially true since all such sulphonic acids might be expected also to resemble one another in other physical properties, such as solubilities. Under these circumstances resort was had to a special form of solubility comparison. Portions of absolute ethyl alcohol were saturated with (*a*) the natural sulphonic acid, (*b*) the synthetic and (*c*) both synthetic and natural. The intensities of ultra-violet absorption of these three solutions were then compared and found to be identical throughout the range of frequency. If the substances were not identical, solution (*c*) should have absorbed with an intensity approximating that of (*a*) + (*b*) In making such comparisons, it was found essential to clarify the solutions thoroughly This was done by centrifuging in a horizontal position in the optical cells in which solution had been effected so that any sediment deposited on the cylindrical walls. It was of course necessary to check carefully the equality of intensity of illumination produced by the two parallel beams of light."

The synthesis of aneurin is shown in outline on pp. 150-1.

Chemical methods for the determination of aneurin are only moderately satisfactory. In alkaline solution potassium ferricyanide oxidises the vitamin to the pale-yellow compound thiochrome, characterised by an intense sky-blue fluorescence (Peters, *Nature*, 1935, **135**, 107; Barger, Bergel and Todd, *ibid.*, **136**, 259; Kuhn, *Z. physiol. Chem.*, 1935, **234**, 196; Jansen, *Rec. Trav. Chim.*, 1936, **55**, 1046).



The fluorescence may be estimated using a fluorimeter, and the maximum reaction is produced at p*H* 10 and extracting the thiochrome by means of isobutyl alcohol after two minutes. Excess of ferricyanide destroys the thiochrome (*cf.* Westenbrink and Goudsmit, *Rec. Trav. Chim.*, 1937, **56**, 803; Pyke, *Biochem. J.*, 1937, **31**, 1958; Karrer and Kubli, *Helv. Chem. Acta*, 1937, **20**, 369).

Colour tests for vitamin B_1 have been suggested by Prebluda and McCollum and by Naiman (*Science*, 1936, **84**, 488, **85**, 290). The detection and estimation of aneurin by chemical methods is, however, a problem requiring much more study.

RIBOFLAVIN

Whey and egg white contain a water-soluble yellow pigment now called riboflavin. The substance was known 60 years ago (Blyth, *J. Chem. Soc.*, 1879, **35**, 530), and Bleyer and Kallmann (*Biochem. Z.*, 1925, **155**, 54) prepared it in a state approaching purity, but the time was neither ripe for

the elucidation of its structure nor the recognition of its importance. The starting point for the recent rapid advances was the work of Warburg and Christian (*Biochem. Z.*, 1932, **254**, 438 ; 1933, **266**, 377 ; *Naturwiss.*, 1932, **20**, 980).

These authors obtained from yeast a new enzyme which in aqueous solution was yellow, with a green fluorescence. The yellow enzyme is easily reduced to its " leuco " form, and under certain conditions the oxidation and reduction of the enzyme is responsible for the transfer of

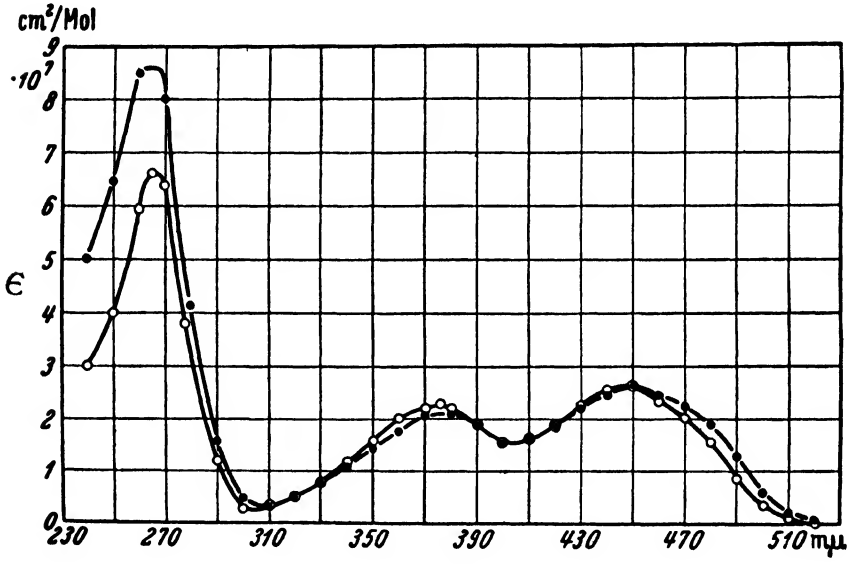


FIG. 57

—o—o— Absorption spectrum of lactoflavin (riboflavin).
—•—•— ,, ,, of alloxazine-adenine nucleotide (see p. 195).

After Warburg and Christian (*Biochem. Z.*, 1938, **298**, 164)

molecular oxygen to substrates. The enzyme seems to occur in nearly all cells, and its functions may be illustrated by the oxidation of Robison's hexose monophosphoric acid ester in the presence of a second yeast enzyme and a coenzyme from horse red blood cells (see p. 187). On addition of three volumes of methyl alcohol at 38° to an aqueous solution of the enzyme, denaturation occurs and protein is precipitated, leaving a pigment in solution. The enzyme is thus seen to be a protein-pigment complex, neither component alone showing the catalytic property of the enzyme. The pigment is the prosthetic group of the enzyme in the sense that it is the site of the reversible oxidation-reduction process. Warburg and

Christian also found that an alkaline solution of the pigment component readily undergoes photochemical decomposition, and a new compound, later known as lumiflavin, was isolated after acidifying the irradiated solution. This compound proved to be a convenient starting point for the elucidation of structure.

Meanwhile, independent researches in several laboratories were providing new evidence of an association between yellow, water-soluble fluorescent pigments and vitamin B_2 (Ellinger and Koschara, *Ber.*, 1933, **66**, 315, 808; Booher, *J. Biol. Chem.*, 1933, **102**, 37; Kuhn, Györgyi and Wagner-Jauregg, *Ber.*, 1933, **66**, 317, 576, 1950; Karrer, Salomon and Schöpp, *Helv. Chem. Acta*, 1934, **17**, 419, 735, 1013). Pigments similar to the coloured component of the yellow enzyme could be obtained from milk, eggs, liver, kidney, urine, muscle and yeast. Kuhn *et al.* proposed the general designation flavin, thus—ovoflavin, lactoflavin, hepatoflavin, etc. The present view is that there is only one natural flavin, *riboflavin*, the name indicating a ribose residue attached to the yellow chromophoric grouping. It is true that synthetic flavins containing sugar groups other than *d*-ribose share with the natural flavin some vitamin B_2 activity, but it is almost certain that riboflavin is more potent than any analogue so far tested.

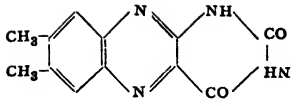
The general scheme for preparing riboflavin involves: (*a*) extraction (aqueous acid, or alcohol-water mixtures), (*b*) adsorption on fullers' earth or lead sulphide (solutions slightly acid), (*c*) elution using pyridine, methyl alcohol and pyridine, or aqueous ammonia, (*d*) removal of eluting solvent, (*e*) precipitation of impurities by addition of acetone to an aqueous solution of the eluate, (*f*) precipitation of the flavin as a salt of a heavy metal (silver and thallium salts are relatively insoluble and easily recrystallised), (*g*) recovery of the flavin, followed by recrystallisations from dilute acetic acid or aqueous alcohol. The riboflavin so obtained forms clusters of yellow needles darkening at 240° (decomp. 274–282°). Analysis indicates $C_{17}H_{20}N_4O_6$, mol. wt. 376; and a tetracetyl derivative, *m.p.* (decomp.) 238–242°, can be prepared.

Properties of Riboflavin

Optical activity very small in neutral or acid solution, $[\alpha]_D^{20}$ —114° in N/10 NaOH (1 mol NaOH per mol. riboflavin).

Irradiation: (*a*) in neutral or acid solution yields 6,7-dimethyl alloxazine, or lumichrome (Karrer *et al.*, *Helv.*, 1934, **17**, 1010) characterised by a sky-blue fluorescence; (*b*) alkaline solution yields lumiflavin.

Riboflavin is stable to H_2O_2, Br_2 water, conc. HNO_3 but is easily reduced. It shows a yellow-green fluorescence in neutral solutions, the best effect being shown at p*H* 6-7.



$\lambda_{max.}$255, 360 mμ

Lumichrome

irradiation
weakly acid
air not

↑ (daylight)
or neutral solution
excluded.

Reduction ($Na_2S_2O_4$ or Pt, H_2).

Riboflavin ⇌ **leucoriboflavin**

oxidation (air)

irradiation of
aqueous solution
air excluded.

leuco-deuteroriboflavin

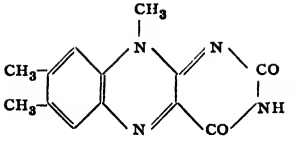
oxidation
(air).

deuteroriboflavin

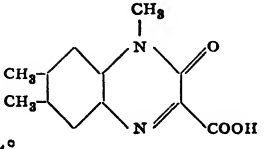
alkali, in the cold and
in the dark.

Lumiflavin

2N. NaOH(hot) or
$Ba(OH)_2$.



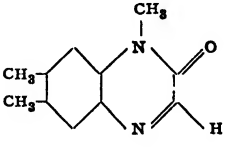
m.p. 321°



+ $(NH_2)_2CO$

m.p. 204°.

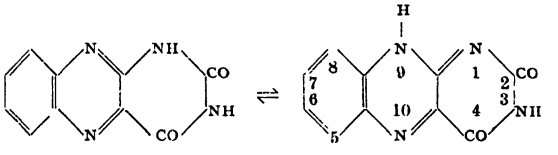
heat
(sublimation)



m.p. 169°.

The structure of riboflavin (or lactoflavin as it was at first designated) was elucidated through the study of photo-decomposition products. The derivative lumiflavin, $C_{13}H_{12}N_4O_2$, is soluble in chloroform but not in water, it does not yield an acetyl derivative, and unlike riboflavin which gives 0·775 mol. formaldehyde on treatment with plumbic acetate, it yields no volatile aldehyde. Its absorption spectrum is so similar to that of riboflavin that the eliminated portion of the molecule cannot be chromophoric. The difference, $C_{17}H_{20}N_4O_6$ minus $C_{13}H_{12}N_4O_2 = C_4H_8O_4$, supports the indications that the group $-(CHOH)_3CH_2OH$ is split off as a result of irradiation, and this is consistent with loss of water-solubility. Kuhn and Rudy (*Ber.*, 1934, **67**, 892) showed that by the action of alkali on riboflavin or lumiflavin, urea and an acid $C_{12}H_{12}N_2O_3$, may be obtained. The latter yields a bright yellow silver salt and is monobasic. The fact that two molecules of water are needed, per molecule of urea obtained by hydrolysis, shows that the $NH_2 - CO - NH_2$ comes from a ring system, rather than from a ureide or a guanidino group in a side chain, where one water molecule only would be required. Hence the ring system furnishes, on cleavage, one oxygen for a carboxyl group and one for the urea molecule. The acid $C_{12}H_{12}N_2O_3$ also contains an alkylimide group $-NCH_3$ which is lacking in riboflavin, so that a sugar residue must have been replaced by the methyl group.

These results suggested a relationship with the alloxazines first described by Kuhling (*Ber.*, 1891, **24**, 2363) with a probability that lumi-



alloxazine *iso*-alloxazine

flavin, with only one active hydrogen (at position 3) is a 6,7-dimethyl-N-methyl-*iso*-alloxazine. The acid undergoes further decomposition when strongly heated and loses CO_2, giving $C_{11}H_{12}N_2O$ (*cf.* Stern and Holiday, *Ber.*, 1934, **67**, 1442; Kuhn, Rudy, Reinemunde and Weygand, *Ber.*, 1934, **67**, 1298, 1459, 1460).

Karrer, Salomon, Schöpp, Schlittler and Fritzsche (*Helv. Chim. Acta*, 1934, **17**, 1010) isolated *lumichrome* from the photo-decomposition products resulting from irradiation in neutral or weakly acid dilute methyl alcohol. Lumichrome, $C_{12}H_{10}O_2N_4$, is a pale yellow crystalline solid which is almost colourless in neutral or acid solution but becomes deep yellow in aqueous ammonia. Solutions of lumichrome in alcohol or chloroform exhibit an

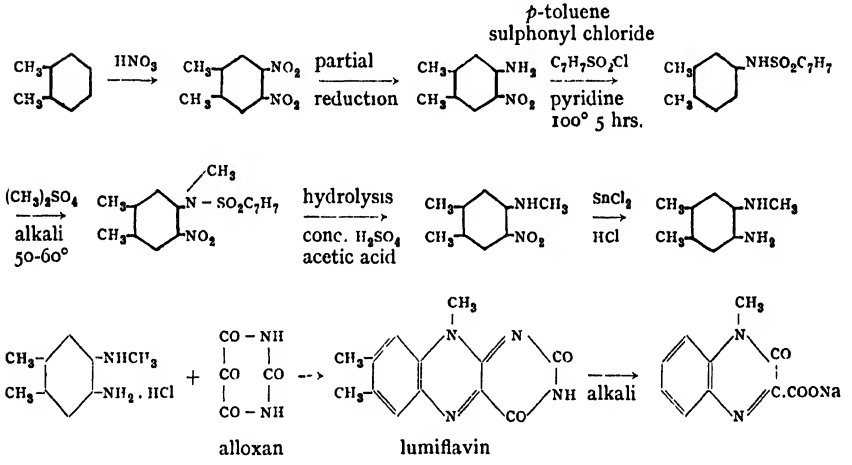
intense sky-blue fluorescence. The properties of lumichrome suggest a dialkyl alloxazine (see p. 155) rather than an *iso*-alloxazine. The compounds

alloxazines		*fluorescence*
6,7-dimethyl alloxazine	- - -	sky-blue
6,8-dimethyl ,,	- - -	blue-green
5,8-dimethyl ,,	- - -	yellow-green

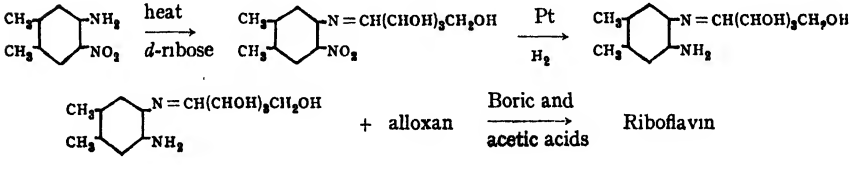
were synthesised and the first was found to be identical with lumichrome.

The irradiation products also gave weak pentose reactions. Synthesis presented relatively little difficulty once the structure had been ascertained.

The following steps are largely self-explanatory:



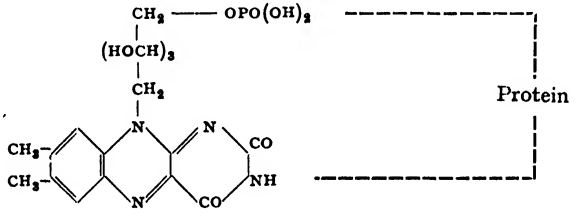
Having established the identity of the lumiflavin with the synthetic product, the nature of the pentose side chain could be tackled. The natural flavin showing vitamin activity, is laevorotatory in alkaline solution. Out of the eight possible stereoisomers, the choice is narrowed to *l*-arabinose, *d*-xylose and *d*-ribose residues, for the hydroxylic side chain. Full physiological activity is obtained with a product synthesised as follows:



A corresponding synthesis with *l*-arabinose yields *l*-araboflavin showing 30–50% activity as compared with riboflavin. Limited supplies of *d*-ribose represent the greatest source of difficulty; the usual method of preparation is from calcium *d*-gluconate, the final yield of *d*-ribose being about 10 g. per kg. of calcium salt. The intermediate *d*-arabinose is much more accessible.

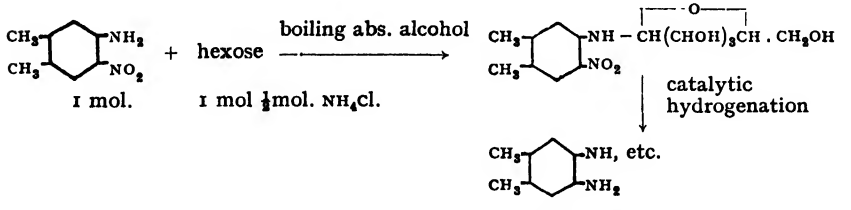
The yellow enzyme has been isolated in the form of pure crystals by Theorell (*Biochem. Z.*, 1934, **272**, 155, **275**, 37), using both cataphoresis and fractional precipitation with ammonium sulphate as methods of eliminating impurities. Union between protein and the prosthetic group is not very strong, because a separation can be effected by dialysis against dilute hydrochloric acid followed by dialysis against water. If solutions (free from electrolyte) of the protein moiety and the flavin respectively are mixed in the cold, enzymic activity is very largely restored. The protein has a molecular weight of the order 70–80,000. The prosthetic group in the enzyme is not riboflavin but its phosphoric acid ester.

Kuhn (*Ber.*, 1936, **69**, 1974, 2034) synthesised riboflavin 5′-phosphoric ester



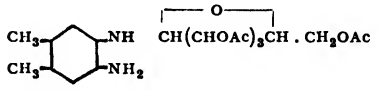
and found it to be identical with cytoflav from heart muscle. The enzyme of yeast may be written as above, because riboflavin and protein do not form an enzymic complex, and combination can be inhibited by blocking the free hydrogen of the imide group.

A group of papers by Kuhn and his colleagues (Kuhn and Ströbele, *Ber.*, 1937, **70**, 747, 753, 773; Kuhn and Cook, *ibid.*, 761; Kuhn and Weygand, 769) greatly clarified the chemistry of the *iso*-alloxazine derivatives. The following process occurs without difficulty:

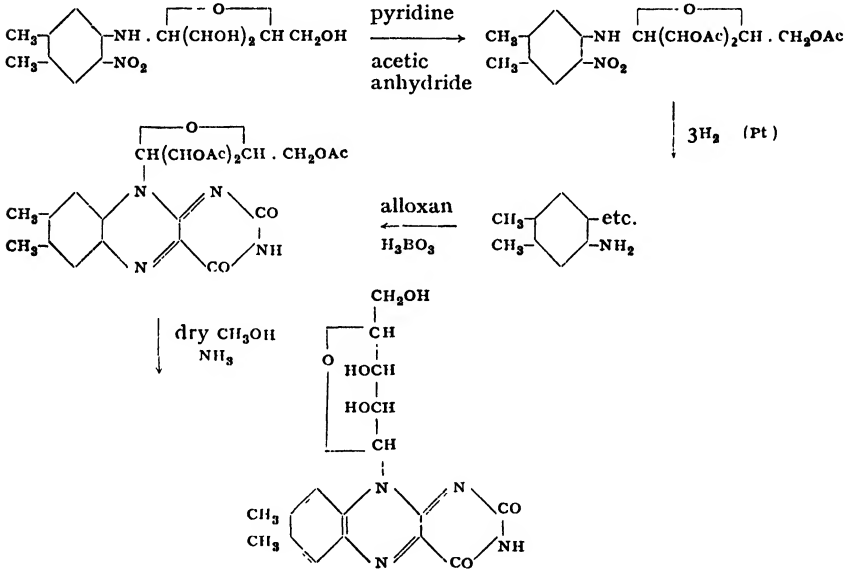


but *o*-phenylenediamines will not condense with reducing sugars, and *o*-phenylenediamine glucosides fail to react smoothly with alloxan to give

flavin derivatives. If however the nitroxylidine glucosides are acetylated (acetic anhydride and pyridine), the triacetyl glucosides may be catalytically reduced to



which condenses readily with alloxan and boric acid to give acetylated flavin glucosides. These may be converted to the flavin glucosides by *dry* methyl alcoholic ammonia. Using these results as a guide to work with the far less accessible *d*-ribose the following synthesis was effected:



6,7-dimethyl, 9, *d*-ribosidoflavin.

This compound resembles riboflavin in absorption and fluorescence; it differs in crystalline form, optical rotation, and is neither active in restoring growth in rats on a B_2 free diet, nor as the prosthetic group of an enzyme. It certainly seems that the rat is incapable of reducing the *d*-ribosido residue to the *d*-ribityl group

6,7-dimethyl 9-*l*-arabityl flavin	$[\alpha]_D^{20}$	$= -25°$	(pyridine)
6,7-dimethyl 9-*l*-arabinosido- ,,	,,	$= 420°$	,,
6,7-dimethyl 9-*d*-riboflavin	,,	$= -30°$	,,
6,7-dimethyl-9-*d*-ribosidoflavin	,,	$= 470°$	,,

Biological activity (rat test, vitamin B_2) is shown by

6,7-dimethyl-, 7-methyl-, 6-methyl- and 6-ethyl, 7-methyl-9[*d*, 1′-ribityl] *iso*-alloxazine and by 6,7-dimethyl-9(*l*- 1′ arabityl) *iso*-alloxazine. Specificity is thus not absolute, but 6- or 7-methyl appears to be essential.

The flavins are of course the basis of important oxidation-reduction systems, and in this connexion the work of Kuhn and Ströbele (*loc. cit.* 753) is of great interest. Riboflavin may be reduced by means of sodium hydrosulphite at p*H* 4–8, giving a green colour, and in strongly acid solution a red colour. The following stages are postulated :

Flavin ⇌ Verdoflavin ⇌ Chloroflavin ⇌ Rhodoflavin ⇌ Leucoflavin

With 6,7-dimethyl-9(*l*-arabityl)-flavin the solubilities are such as to facilitate isolation more readily than with riboflavin.

All the reduced compounds revert quantitatively to the flavin on shaking with oxygen in N/10 sodium hydroxide, and manometric determination of oxygen uptake permits the degree of reduction to be ascertained.

Determination of riboflavin

Kuhn, Wagner-Jauregg and Kaltschmitt (*Ber.*, 1934, **67**, 1452) described a spectrophotometric method for determining the amounts of flavin occurring in natural products. They found it convenient to convert the riboflavin to lumiflavin and to use the wave-length 470mμ, determining the intensity of absorption according to a standard procedure. It is perhaps desirable to use pure lumiflavin as the standard for comparison and to multiply by 1·5 (*i.e.* a factor corresponding approximately with the ratio of molecular weights : riboflavin/lumiflavin = 376/256).

RIBOFLAVIN CONTENT OF FOODSTUFFS

Product	*mg. lumiflavin*	*mg. riboflavin*
apple juice (1 l.)	0·059	0·089
light beer (1 l.)	0·19	0·29
cow's milk (1 l.)	0·67	1·00
dried apricots (1 kgm.)	0·38	0·57
dried spinach (1 kgm.)	3·80	5·70
fresh grass (1 kgm.)	0·95	1·42
yeast (1 kgm.)	12·0	18·0
marmite (1 kgm.)	22·0	33·0
egg albumen (dry) (1 kgm.)	9·4	14·1
fresh ox liver (1 kgm.)	10·6	15·9

Hodson and Norris (*J. Biol. Chem.*, 1939, **131**, 621) determine riboflavin fluorimetrically, but the methods described by them are useful in other ways:

The material (5 g) is weighed into a flask (0·5 l.) and N/4 H_2SO_4 (50 ml. or 100 ml. for bulky products) added and the mixture heated gently under reflux (1 hr.). On cooling, Na_3PO_4 solution is added to bring the p*H* to 7·0–7·5. Water is added (50 ml) and after ½ hr. the solution is filtered. An aliquot part (depending on riboflavin content) of the filtrate is diluted to *ca* 175 ml. and hydrosulphite (2 ml. of a solution in 20 ml. ice-cold water of Na_2SO_4, 1 gm., $NaHCO_3$ 1 g.) and stannous chloride (2 ml., of a solution $SnCl_2$, 10 g., conc. HCl 25 ml., diluted 200-fold with water) added and the solution made up to known volume. Reduction is complete in 10 mins., and the solution is then shaken with air in a larger (1 l.) flask for 5 mins. Riboflavin is re-oxidised, but interfering pigments and fluorescent substances remain in the reduced state. A definite volume of solution is placed in a suitable cell and the fluorescence determined in a fluorimeter.

At this stage the procedure is subjected to a " calibration " control: (i) a known quantity of pure riboflavin is added to the unknown solution, (ii) another portion of the unknown solution is reduced (2% of its volume of $Na_2S_2O_4$ solution) and (iii) a solution containing only the pure riboflavin as added in (i). Fluorescence analysis of (i) and (iii) gives the true riboflavin content, indicated by the difference between the apparent riboflavin contents before and after reduction. Interfering pigments make it necessary to use a correction factor obtained by multiplying by:

riboflavin content of standard solution/difference contents before and after addition of flavin to the unknown.

Milk products like casein are exttracted by means of acetone (3 vols.) and N . H_2SO_4 (1 vol.) rather than N/4 H_2SO_4. With highly coloured materials like molasses, interfering pigments may be removed by neutralising the extract and adding methyl alcohol (10 vols.) slowly to an aliquot part of the neutralised extract. After filtration, the usual procedure is followed. Some flavin may adhere to the methyl alcohol precipitate and the method should always be checked by determining the percentage recovery of added riboflavin.

The best sources of riboflavin according to this method are:

	p.p.m.(*mg./kgm.*)
liver meal	*ca* 80
dried yeast	30–50
dried whey	20
dried alfalfa meal	15
white fish meal	10

and the results are consistent with biological assays.

Nicotinamide

Surprise is often expressed that the importance of nicotinamide escaped recognition for so long in spite of the fact that it was one of the earliest substances to be isolated from extracts containing water-soluble vitamins. There is now no doubt that the substance plays a special part in the prevention of pellagra and canine blacktongue, but a general review of the relationship between pellagra and diet, pellagra and light (Sebrell, *J. Amer. Med. Assoc.*, 1938, **110**, 1665), shows that the whole subject is extremely difficult. Much careful work was needed, for instance,

to establish that neither riboflavin nor vitamin B_6 was identical with the P–P and anti-black-tongue factor, and as always such negative results were only a shade less important than the more positive findings which emerged later.

So far as absorption spectra are concerned, the discussion of nicotinamide is best deferred to the section on co-enzymes (see p. 186).

The most convenient method of determining the nicotinamide content of tissue is based on the formation of a yellow substance by the action of alkali on the product obtained by fusing nicotinamide with 2,4–dinitrochlorobenzene (Karrer and Keller, *Helv. Chim. Acta*, 1939, **22**, 1292 ; *cf.*

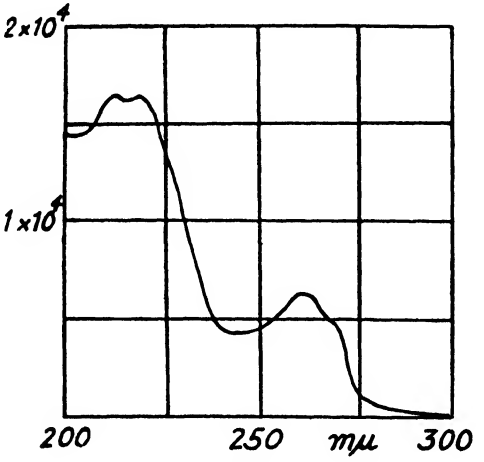


FIG. 58
Nicotinamide in water
After Kuhn and Vetter

ibid., 1938, **21**, 463, 1170 ; Euler *et al.*, *Z. physiol. Chem.*, 1939, **258**, 212).

The finely minced tissue, mixed with three times its volume of water, is boiled for thirty minutes. The operation is carried out again with two fresh portions of water and the united extracts are shaken three times with a little ether to remove fatty material. This solution contains nicotinamide as part of a co-enzyme (p. 188). The solution is acidified (H_2SO_4) until it is decinormal, and is allowed to simmer for thirty minutes. This decomposes the co-enzyme. A hot solution of barium hydroxide is now added until the barium sulphate precipitation is complete (faintly alkaline towards litmus) ; the solid is removed and washed twice with hot water. The filtrate and washings are then placed in a continuous extractor and after five or six hours of treatment with butyl alcohol all the nicotinamide has been extracted. The residue obtained after removal

of the butyl alcohol is then boiled three times with small portions of water and the extract filtered if necessary. It is then evaporated to complete dryness under reduced pressure (addition of absolute methyl alcohol and dry benzene facilitates drying).

The quaternary pyridinium compound is prepared by adding 2,4-dinitrochlorobenzene (three times the weight of solid residue) in methyl alcohol. The solvent is removed and the dry mixture of nicotinamide and the dinitro-body are maintained for thirty minutes in a molten state on a water bath. The product is dissolved in 80% alcohol, made up to known volume, and a few drops of 10% alcoholic potash are added to a suitable volume of solution. The yellow colour develops and the intensity may be determined, using such apparatus as may be available for accurate colorimetry.

NICOTINAMIDE CONTAINED IN FRESH TISSUES

	p.p.m. (*mg./kgm.*)	
Muscle (rat)	50·8	(Karrer and Keller, *loc. cit.*)
(horse)	46·6	
(ox)	38·3	
Liver (horse)	160	
(ox)	93	
Kidney (ox)	194	
Baker's yeast	120	

Vitamin H

Györgyi gave the name vitamin H, and Boas the designation " protective factor H ", to a substance which prevents the appearance of a characteristic skin disorder in rats on a diet rich in egg white (egg white injury). The real existence of this vitamin is evidenced in recent papers by Györgyi, Kuhn, Lederer and Birch (*J. Biol. Chem.*, 1939, **131**, 733, 745, 761). The toxic effect of egg white disappears on digestion with HCl or pepsin and on prolonged heat treatment. Vitamin H occurs in yeast, liver and kidney, but cannot easily be extracted. It is best obtained by heating liver or kidney with aqueous acid under pressure (200°).

Considerable progress has been made in preparing concentrates by chemical methods and by electrodialysis. Vitamin H is an ampholyte with an *iso*-electric point between p*H* 3 and 3·5. A barium salt is precipitated from alcohol at p*H* 5 and the most active preparations contain neither sulphur nor phosphorus.

Vitamin B_6 (Pyridoxin, or Adermin).

In 1926, Goldberger and Lillie described a nutritional dermatitis in rats, and in 1934 P. Györgyi (*Nature*, **133**, 498) used the designation vitamin B_6 for the factor which prevents the appearance of the condition. Rat acrodynia, as it is sometimes called, is a syndrome not unlike pellagra, and is characterised by a symmetrical dermatosis affecting primarily the paws, the tips of the ears and the nose. An analogous deficiency dermatitis may be induced in chicks. Various workers on the B complex have independently arrived at the concept of a factor with functions related to

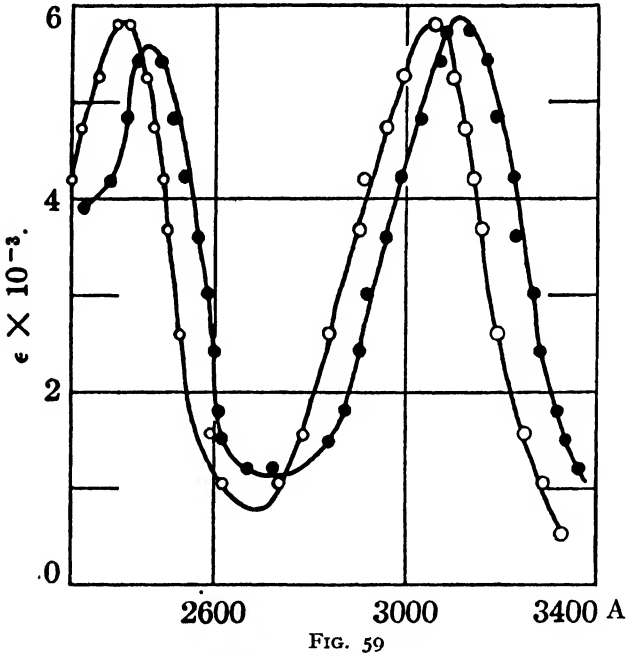


FIG. 59

Absorption spectra in aqueous solution at p*H* 10·2 for ●—●—●— vitamin B_6.
o—o—o— 2-methyl-3-hydroxy-5-ethylpyridine.
After Stiller *et al.*

those of vitamin B_6. Thus the γ factor of Chick and Copping, vitamin H of Hogan and Richardson, of Booher and of Kuhn, and the Factor I of Lepkovsky and Jukes, all have a good deal in common with B_6. Progress in vitamin chemistry has resulted in many surprises and it would be very incautious to conclude that all the above factors are merely synonymous with vitamin B_6. It is, however, safe to say that they will need to be carefully differentiated from this substance. The vitamin has recently been isolated, characterised, synthesised and its biological activity verified. At first it was known as *adermin*, but *pyridoxin* is more widely used.

When rice, bran, yeast, wheat germ or fish muscle is extracted, the B_6 factor is found to be water-soluble. After removal of vitamin B_1 and riboflavin, the extract is treated with a large quantity of fuller's earth at p*H* 2·5–5·0. Vitamin B_6 is adsorbed but the filtrate-factor (nicotinamide, effective in the relief of canine black-tongue and human pellagra), remains in solution. Vitamin B_6 is also adsorbed on activated charcoal, from which it may be eluted by means of *n*-butyl alcohol (Birch and Györgyi, *Biochem. J.*, 1936, **30**, 304; Halliday and Evans, *J. Biol. Chem.*, 1937, **118**, 255, Booher, *J. Biol. Chem.*, 1937, **119**, 223). The concentrate behaves

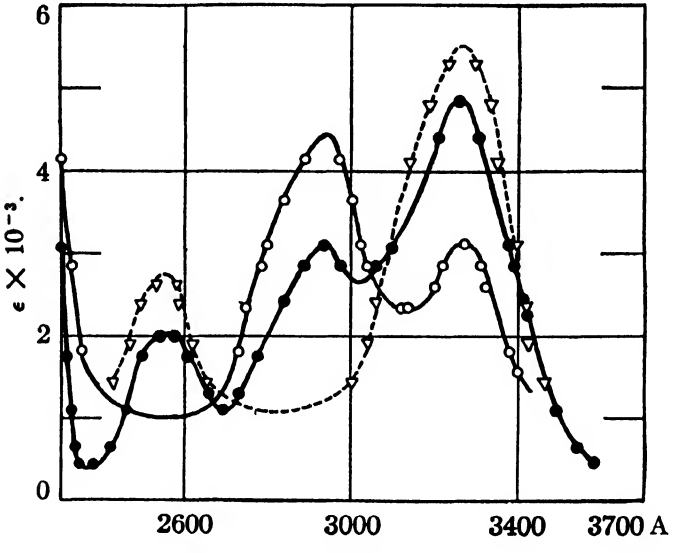
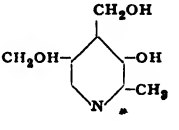


FIG. 60

Absorption spectra of vitamin B_6 at:
o—o— p*H* 4
●—●— p*H* 5·1
—▽—▽— p*H* 6·75
After Stiller *et al.*

as a dialyzable substance of low molecular weight, stable to heat and to alkali and not very soluble in water. It is carried down by phosphotungstic acid, easily adsorbed on kieselguhr and destroyed by ultra-violet irradiation. Not much is known concerning its physiological functions other than those connected with the prevention and cure of rat acrodynia but promising work is in progress. Pyridoxin (or adermin) was isolated from rice bran as the hydrochloride $C_8H_{12}O_3N.Cl$, *m.p.* 204–206° (dec.), of a base $C_8H_{11}O_3N$, *m.p.* 160°, possessing the structure

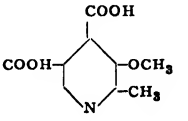


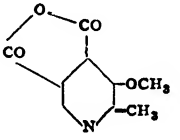
(2-methyl,3-hydroxy,4,5-di-(hydroxymethyl)-pyridine (Keresztesy and Stevens, *J. Amer. Chem. Soc.*, 1938, **60**, 1267; Keresztesy and Stevens, *Proc. Soc. Expt. Biol. Med.*, 1938, **38**, 64; Stiller, Keresztesy and Stevens, *J. Amer. Chem. Soc.*, 1939, **61**, 1267; Harris, Stiller and Folkers, *ibid.*, 1242; Harris and Folkers, *ibid.*, 1245; Ichiba and Michi, *Sci. Papers Inst. Phys. Chem. Res.*, 1938, **35**, 73; 1939, **36**, 173; Kuhn and Wendt, *Ber.*, 1939, **72**, 305, 311; Kuhn *et al.*, *ibid.*, 310; Györgyi, *J. Amer. Chem. Soc.*, 1938, **60**, 983).

The following facts are adduced in support of the above structure:

(*a*) the compound forms a hydrochloride and a monomethyl ether.

(*b*) the ether, on oxidation (CrO_3 and H_2SO_4), yields rather less than 1 mol. of acetic acid and with neutral permanganate gives a pyridine dicarboxylic acid of structure



The lactone  is an intermediate product.

(*c*) the methyl ether of "B_6" does not undergo oxidation with lead tetracetate. Both alcoholic hydroxyls are free and the compound is not an α-glycol.

(*d*) the vitamin contains one C-methyl group, three active hydrogens, and gives a red colour with $FeCl_3$ similar to that produced by β-hydroxy-pyridine.

(*e*) It is a weak base the pK_b of which is of the same order as that of β-hydroxy-pyridine.

The absorption spectra shown by pyridoxin and related substances are of great interest. Figs. 59 and 60 are reproduced from the work of Stiller, Keresztesy and Stevens (*loc. cit.*). The vitamin is a tautomeric compound the absorption of which depends markedly on p*H*. In this it greatly resembles

2-methyl,3-hydroxy,5-ethyl-pyridine
2-methyl,5-hydroxy-pyridine and
β-hydroxy-pyridine

In acid solution (p*H* 2·1) all four compounds show a single unresolved band ($\lambda_{max.}$ 292mμ except for β-hydroxy-pyridine, 282·5mμ). In alkaline

solution this band disappears and is replaced by two new bands (Figs. 61, 59). The spectra shown by the vitamin at intermediate values illustrate very clearly the gradual nature of the replacement (Fig. 60).

The differences between the spectra of β-hydroxy-pyridines and α- and γ-pyridones are very marked and there is no room for doubt as to the diagnostic value of spectrophotometry in this instance.

It is interesting that Kuhn and Wendt's adermin monomethyl ether (B_6 plus diazomethane) shows a single band, $\lambda_{max.}$ 280mμ, $\epsilon_{max.}$ 5000, un-

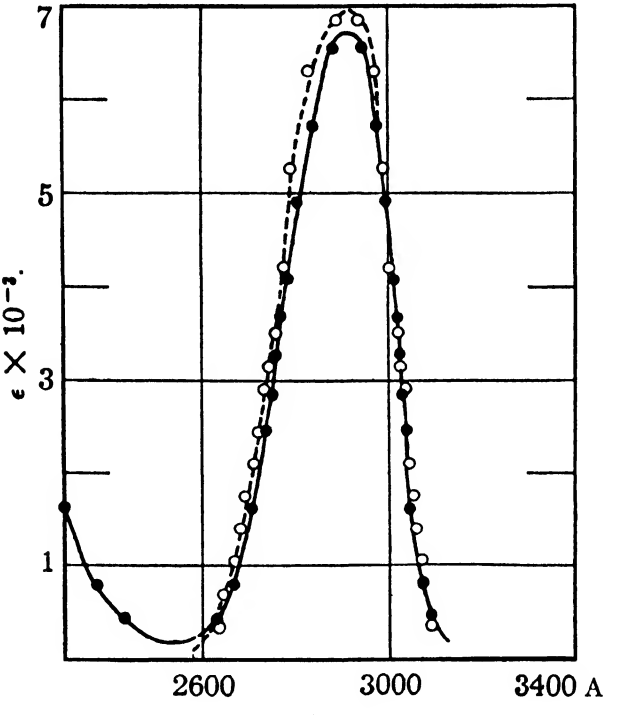


FIG. 61

Aqueous solutions at pH 2·1

—●—●— vitamin B_6.

—○—○— 2-methyl-3-hydroxy-5-ethyl pyridine.

After Stiller *et al.*

influenced by change of pH. When this product is oxidised by $Ba(MnO_4)_2$ the dibasic acid already referred to is obtained, *m.p.* 208–209°. The spectrum shows a marked resemblance to that of 2, 6-dimethyl cinchomeronic acid (Fig. 62).

Harris and Folkers (*loc. cit.*) and Kuhn *et al.* (*loc. cit.*) completely synthesised the vitamin and found it to be chemically and physiologically

identical with the product isolated from natural sources. A single dose of 100 mg. proves sufficient to effect a complete cure in 14 days when fed to vitamin B_6-deficient rats.

Bios

In 1860 Pasteur (*Ann. Chim. Phys.* III, **58**, 323) published his memoir on alcoholic fermentation and claimed that yeast could be grown in a medium containing only yeast ash, an ammonium salt and a fermentable sugar. Liebig (*ibid.*, 1869, IV, **23**, 5) dissented vigorously and Pasteur (*ibid.*, 1872, IV, **25**, 145) refused emphatically to withdraw. In 1901

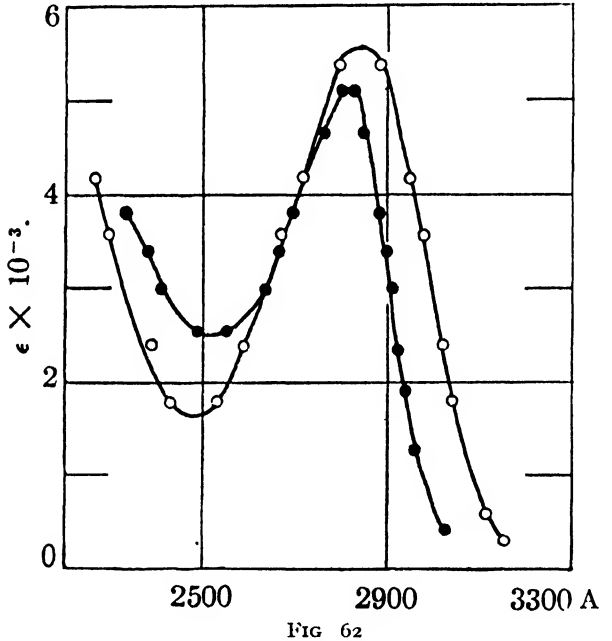


FIG 62

—●—●— dibasic acid $C_9H_9O_5N$.

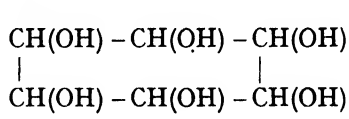
—○—○— 2,6-dimethylcinchromeronic acid.

After Stiller *et al.*

Wildiers (*Cellule*, **18**, 313) found that in order to start a new culture the presence in small amount of organic substances extractable from yeast was necessary. He used the term " bios " to describe material contained in yeast and necessary for its growth, but the concept had a mixed reception. Lash Miller (*Science*, 1934, **59**, 197) and Lucas (*J. Phys. Chem.*, 1924, **28**, 1180) stressed the fact that different yeast species vary markedly in their growth responses, and Miss Copping (*Biochem. J.*, 1929, **23**, 1050) confirmed this using twenty species. The wild yeasts are active in respiration but do not require bios in order to grow on a synthetic medium,

whereas the cultivated yeasts need added bios before they grow normally. Liebig and Pasteur may have made use of different species.

Lucas (*loc. cit.*) showed that " bios " could be separated into fractions, bios I and bios II, by means of alcoholic barium hydroxide or basic lead acetate. Bios I (barium salt insoluble in alcohol) was identified by Eastcott (*ibid.*, 1928, **32**, 1094) as meso-inisitol:



Lucas' bios II is evidently complex. R. J. Williams and his colleagues (*J. Amer. Chem. Soc.*, 1929, **51**, 2764; *J. Biol. Chem.*, 1930, **87**, 581; *J. Amer. Chem. Soc.*, 1931, **53**, 783, 4171; *Biochem. J.*, 1934, **28**, 1887) carried out a great deal of work from which it appeared that (*a*) inisitol plays some part in stimulating the growth of a large number of yeasts. Alone, it is not very effective, but in conjunction with other constituents of the bios complex it may give striking increases at the level 1–10μg/c.c. of medium; (*b*) aneurin can replace part of the bios complex very efficiently in certain yeasts; (*c*) pantothenic acid (p. 170) is effective alone at the level 0·008μg/ml. for all the yeasts and its activity may be enhanced by inisitol or aneurin or both. Pantothenic acid is not adsorbed by fuller's earth, whereas aneurin and other materials are strongly retained.

Farrell (*Trans. Roy. Soc. Canada*, 1935, III, **29**, 167) claims that a bios V exists and is akin to ascorbic acid, and Williams and Rohrman (*J. Amer. Chem. Soc.*, 1936, **58**, 695) regard alanine as a growth essential for yeasts. Kögl and Tonnis (*Z. Physiol. Chem.*, 1936, **242**, 43) obtained from egg yolk a substance, *biotin*, active at a dilution of 1 in 4×10^{11}, but the yield of crystalline material was too small for detailed characterisation to be possible.

1,000 eggs were boiled for 15 minutes, and the yolks (20 kgm.) boiled twice with water (80 l.). After filtration, the extract was diluted with half its bulk of acetone and 149 gm. of inactive material precipitated. The liquor was reduced to dryness *in vacuo* (300 g.), dissolved in water (1 l.) and alcohol (4 l.) added. The precipitate (158 g.) was highly active. Further treatments with lead acetate, mercuric chloride, phosphotungstic acid, brompicrolonic acid, followed by adsorption on animal charcoal and elution with acetone and ammonia, resulted in progressive concentration of the active principle. High vacuum distillation (0·001 mm.) on a micro scale yielded a fraction, *b.p.* 135–185°, showing intense blue fluorescence in ultra-violet light and a main fraction, 185–250°, fluorescing blue-green and intensely active. By adding petrol to the chloroform solution needles, *m p.* 148°, were obtained.

Kögl showed that the extraordinarily great activity of the crystalline material (biotin) was greatly enhanced by the simultaneous presence of inisitol in the crude product. Biotin is probably an acid, it contains

nitrogen but neither sulphur nor phosphorus. Its absorption spectrum has unfortunately not been studied although the characteristic fluorescence suggests that interesting results might be expected.

Recent work on pantothenic acid (Williams, Weinstock and Rohrmann, Truesdail, Mitchell and Meyer; Jukes, *J. Amer. Chem. Soc.*, 1938, **60**, 2719, **61**, 454, 975) indicates that the calcium salt of the pure acid prevents a form of dermatitis in chicks. The following table indicates the present state of knowledge:

Pantothenic acid

$C_8H_{15}O_5N$. M.W. *ca* 200

a monobasic acid from % Ca in calcium salt

amino N negligible (van Slyke)

no amide group (absence of NH_3 on heating with alkali)

no methoxyl groups (micro-Zeisel)

no ethylenic linkages (catalytic hydrogenation; no Br_2 absorbed from $C Cl_4$)

no $-CHO$ or $C=O$ groups (no reduction Adams catalyst, or sodium amalgam); oxidation very difficult, H_2O_2, $KMnO_4$; no reaction $C_6H_5NH . NH_2$)

no aromatic groups (absorption spectrum shows only end absorption).

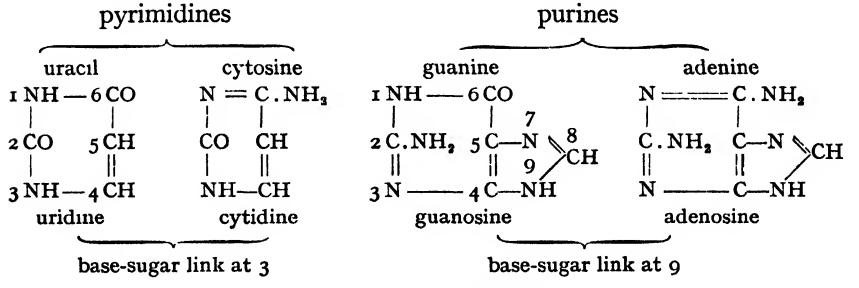
CHAPTER VIII

PURINE AND PYRIMIDINE DERIVATIVES

Nucleic acids

The nucleoproteins are of fundamental importance to all living organisms, and the substances derived from them are closely related to many degradation products of vitamins and hormones. In extreme cases the proportion of protein is quite low, the heads of salmon spermatozoa containing 30·6% protamine and 60·5% nucleic acid. The nucleoprotein of the thymus and the pancreas is richer in protein, but on the other hand nucleic acids occur uncombined in yeast. Very gentle hydrolysis is sufficient to rupture the protein-nucleic acid linkages.

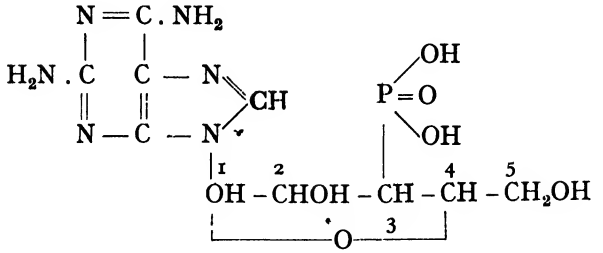
Alkaline hydrolysis in the cold, causes nucleic acids to break down, yielding nucleotides; further hydrolysis (with dilute ammonia) splits off phosphoric acid, yielding nucleosides. Complete hydrolysis effects cleavage of the nucleosides, yielding (in the case of yeast nucleic acid) *d*-ribose or its degradation product furfural, and uracil, cytosine, adenine and guanine. The subject has recently been reviewed by Gulland (*J. Chem. Soc.*, 1938, 1722), and the account presented in this chapter is intended mainly to bring out the points connected with spectroscopic studies and having a direct bearing on related enzyme and vitamin studies. The nomenclature is illustrated in the following diagram:



nucleosides, *i.e.* *d*-ribosides

Nucleotides:

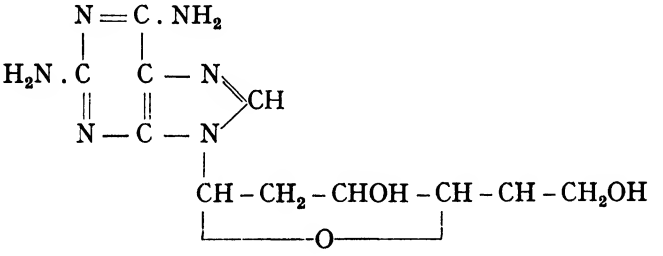
uridylic, cytidylic, guanylic and adenylic acids are phosphoric acid derivatives, the ester link being at C_3.



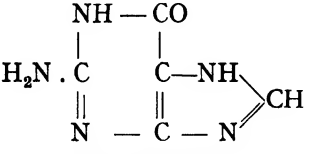
yeast adenylic acid

The manner in which the four nucleotides are united to form nucleic acid presents a very difficult problem; it is clear, however, that the basic components are not concerned (see Gulland, *loc. cit.*).

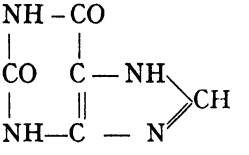
Thymo-nucleic acid (prepared from the thymus gland) differs from yeast nucleic acid in that *d*-2-deoxyribose replaces *d*-ribose so that nucleosides have structures of the type:



The point of attachment of phosphoric acid to the sugar is not yet established. The pancreas contains a ribonucleic acid and a deoxyribonucleic acid. Thymine occurs instead of uracil in products derived from deoxyribonucleic acid and hypoxanthine results from enzymic deamination of adenine:



hypoxanthine



xanthine

Gulland, Holiday and Macrae (*J. Chem. Soc.*, 1934, 1639), taking advantage of the work of Goos *et al.* (*Z. physiol. Chem.*, 1930, **186**, 148), on the transparency of simple carbohydrate groups, applied spectrophotometry to the problem of determining the position of the sugar-base linkage in the purines. Purely chemical investigation had left

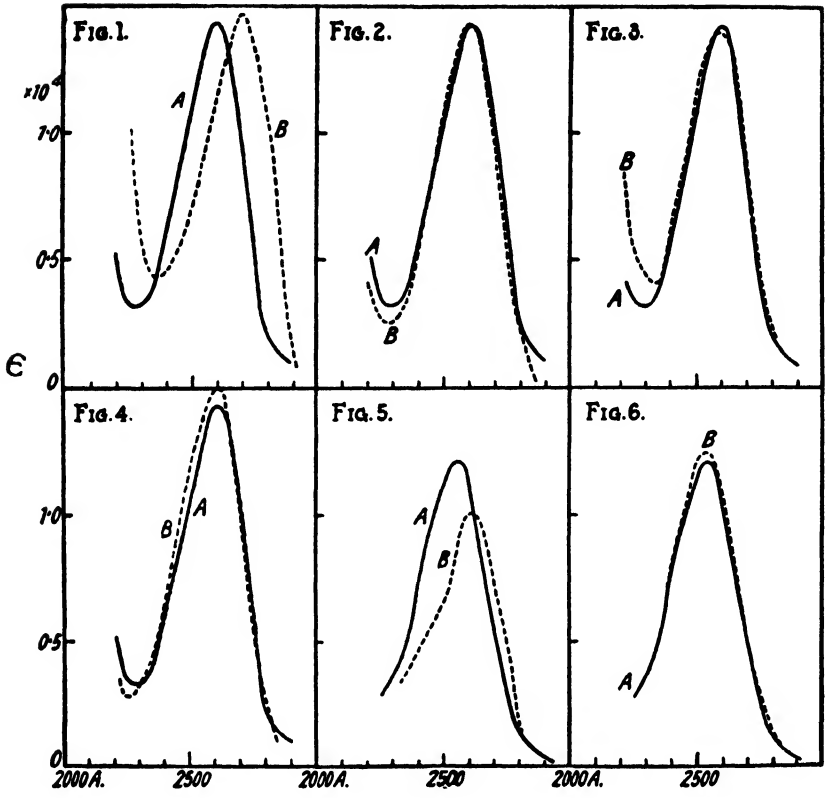


Fig. 63.

1. *A*. 9-methyladenine in water, N/20-NaOH, and N/20-HCl. *B*. 7-methyladenine in water and N/20-HCl.

2. *A*. 9-methyladenine. *B*. Adenosine in water, N/20-NaOH, and N/20-HCl.

3. *A*. 9-methyladenine. *B*. Muscle adenylic acid in water at p*H* 4·5, in N/20-NaOH, and N/20-HCl.

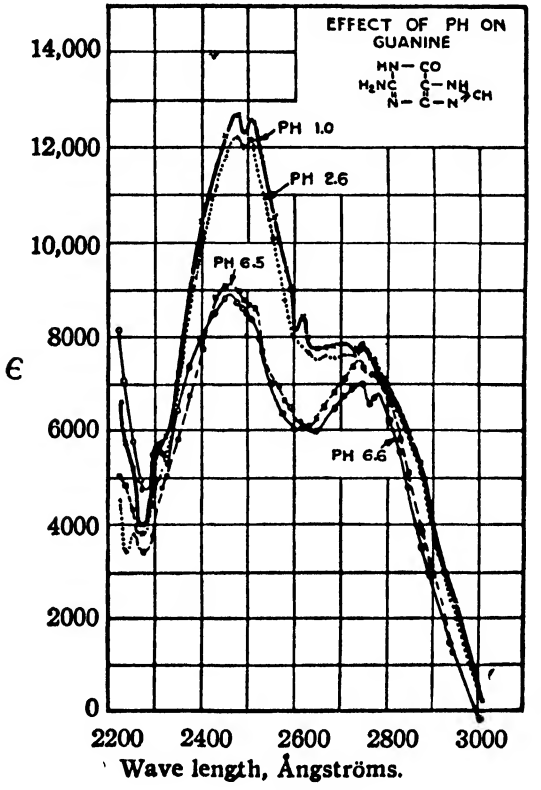
4. *A*. 9-methyladenine. *B*. Barium adenyl pyrophosphate in water at p*H* 6·5, in N/20-NaOH, and N/20-HCl.

5. *A*. 9-methylhypoxanthine in N/20-NaOH. *B*. 7-methylhypoxanthine in N/20-NaOH.

6. *A*. 9-methylhypoxanthine in N/20-NaOH. *B*. Inosine in N/20-NaOH.

After Gulland.

open the possibility of linkage at positions 1, 3, 7 or 9. The four methyl xanthines were therefore prepared and their spectra compared with that of xanthosine.



After Heyroth and Loofbourcw.

FIG. 64

	in HCl	pH 5	in NaOH	pH 10
	$\lambda_{max.}$ mμ	$\epsilon_{max.} \times 10^{-4}$	$\lambda_{max.}$ mμ	$\epsilon_{max.} \times 10^{-4}$
xanthosine	264	0·84	278	0·74
	238	0·78	247	0·86
1-methyl xanthosine	266	1·02	276	0·85
			241	0·85
3- ,, ,,	271	1·0	273	1·2
7- ,, ,,	269	0·96	290	0·97
9- ,, ,,	264	0·93	278	0·93
	235	0·73	247	0·93
caffeine	273	0·97	273	0·97
	267	0·90	269	0·90
iso-caffeine	239	0·76	240	0·76

The spectroscopic evidence makes it certain that the linkage occurs at position 9.

In a series of papers published since (*J. Chem. Soc.*, 1936, 765; 1937, 1912; 1938, 259, 692; 1939, 907), Gulland and his co-workers have fully exploited this application of absorption spectra. Throughout the series of nucleosides it is found that the purine base-sugar linkage occurs at position 9.

TABLE

solvent	N/20 HCl		N/20 NaOH		water	
	$\lambda_{max.}$ mμ	$\epsilon_{max.} \times 10^{-4}$	$\lambda_{max.}$ mμ	$\epsilon_{max.} \times 10^{-4}$	$\lambda_{max.}$	$\epsilon_{max.} \times 10$
adenine	260	1·32	258	1·36		
9-methyl adenine	260	1·42	260	1·47		
7-methyl adenine	269	1·46	269	1·14		
adenosine	260	1·42	260	1·43		
adenylic acid (muscle)	260	1·29	260	1·40		
adenyl pyrophosphate	260	1·49	260	1·38		
adenine thiomethylpentoside	260	1·42	260	1·42		
*adenine deoxyriboside	260	1·6	260	1·75		
adenine *d*-glucoside	260	1·42	260	1·52		
hypoxanthine	248	0·96	262	1·00		
9-methyl hypoxanthine	248	1·03	255	1·21		
7-methyl hypoxanthine	250	0·98	261	1·01		
inosine	247	1·32	254	1·34		
7-methyl guanine	250 ~280	1·02 0·6	~240 282	0·55 0·75		
9-methyl guanine	252 280	1·32 0·66	263	0·92		
guanine deoxyriboside	—	—	261	0·90		
guanosine	253 ~280	1·25 0·7	263	0·92		
xanthosine	234 263	0·8 0·87	258 281	0·92 0·82	240 278	0·86 0·84
9-methyl *iso*-guanine	228 270	0·45 0·98	275 239	0·65 0·42	236 283	0·73 0·76
(crotonoside very similar)						
1-methyl uric acid	260 ~281	0·9 0·5	260 295	0·9 0·7		
3- ,, ,,	234 292	0·8 1·0	292	1·05		
7- ,, ,,	234 287	0·7 0·9	297	0·96		
9- ,, ,,	238 285	0·68 0·78	252 302	1·05 0·78	238 ~252 292	0·75 0·6 0·77

* Probably hydrolysed by acid.

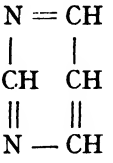
Uric acid riboside from blood and from liver show spectra almost identical with those shown by 9-methyl uric acid.

The pyrimidine-sugar linkage occurs at position 3. This follows from the conversion of cytidylic acid to uridylic acid by nitrous acid, and the hydrolysis of 1-methyl-uridine (from uridine) to 1-methyl-uracil (Levene and Tipson, *J. Biol. Chem.*, 1934, **104**, 385).

Spectrophotometric studies on purines are relevant to the study of coenzymes (Chapter X, coenzymes, etc.), and the data on the pyrimidines

to the problem of the structure of aneurin where further data are given have already been discussed to some extent in connection with the structure of aneurin

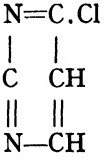
Pyrimidine derivatives

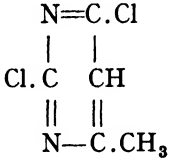
The parent substance  is difficult to obtain in a

high state of purity, but the following data are probably near to the truth:

$\lambda_{max.}$ mμ	239	243·5 ~ 250	feebly resolved absorption 260–295mμ	
$\epsilon_{max.}$	2,650	3,075 1,600	ε *ca* 250–300	

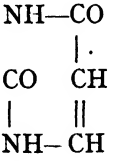
Introduction of substituents displaces the absorption and results in increased ε values:

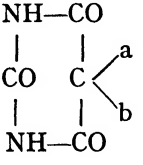




$\lambda_{max.}$ mμ	259	~254	259	~254	~265	
$\epsilon_{max.}$	4,700	4,130	5,450	4,600	4,250	

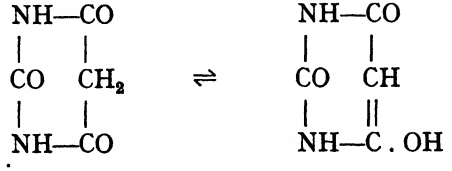
Uracil shows quite intense absorption in the same region but pyrimidine derivatives not containing ethylenic linkages are relatively transparent:





alloxan	a = OH	b = OH, end absorption 260–220mμ	
dialuric acid,	a = H	b = OH	,,
barbital	a = C_2H_5	b = C_2H_5	,, ~265 mμ, ε *ca* 1800

Barbituric acid shows very intense absorption, $\lambda_{max.}$ 260mμ, $\epsilon_{max.}$ 25,000,



the origin of which is a little obscure. The curves for pyrimidine derivatives containing one or two double bonds all exhibit more intense absorption than do those of derivatives with three conjugated double bonds. This phenomenon runs parallel with the cases of cyclohexadiene and benzene (see p. 17).

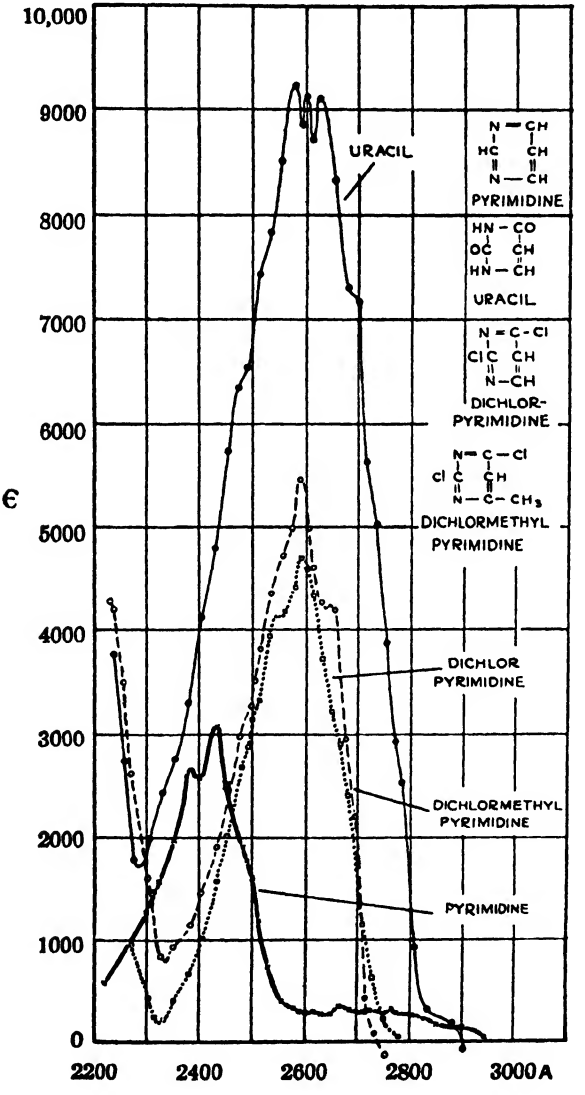


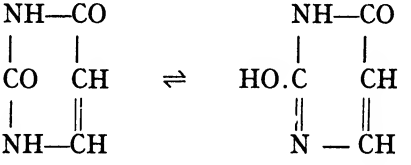
FIG. 65

After Heyroth and Loofbourow.

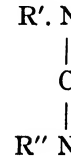
	$\lambda_{max.}$ mμ	$\epsilon_{max.}$
uracil - - -	*ca* 260	*ca* 9,000 (resolved, 257, 260, 264mμ)
thymine - -	263	8,600
cytosine - -	265	6,150
iso-cytosine - -	265	6,150
iso-barbituric acid	280	6,400

(Heyroth and Loofbourow, *J. Amer. Chem. Soc.*, 1934, **56**, 1728).

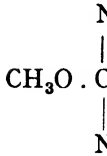
Uracil derivatives may exhibit lactam-lactin tautomerism :



Austin (*J. Amer. Chem. Soc.*, 1934, **56**, 2141) studied a number of substituted products, the structures of which are fairly well established :

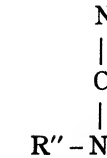


R′ and R″, alkyl groups.
$\lambda_{max.}$ 265mμ ($\epsilon_{max.}$ *ca* 7,000)
(uracil 258mμ $\epsilon_{max.}$ 6,500
1 methyl uracil 265mμ)

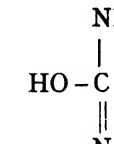


or di-ethoxy homologue (*cf.* dichloropyrimidine)

$\lambda_{max.}$ 258mμ $\epsilon_{max.}$ *ca* 7,000
(3-methyluracil 258mμ)
The absorption is narrower and less persistent than for uracil, and the minimum occurs at longer wave-lengths.



$\lambda_{max.}$ 275mμ $\epsilon_{max.}$ *ca* 5,000



$\lambda_{max.}$ 258mμ $\epsilon_{max.}$ *ca* 700
(note great decrease in ϵ)

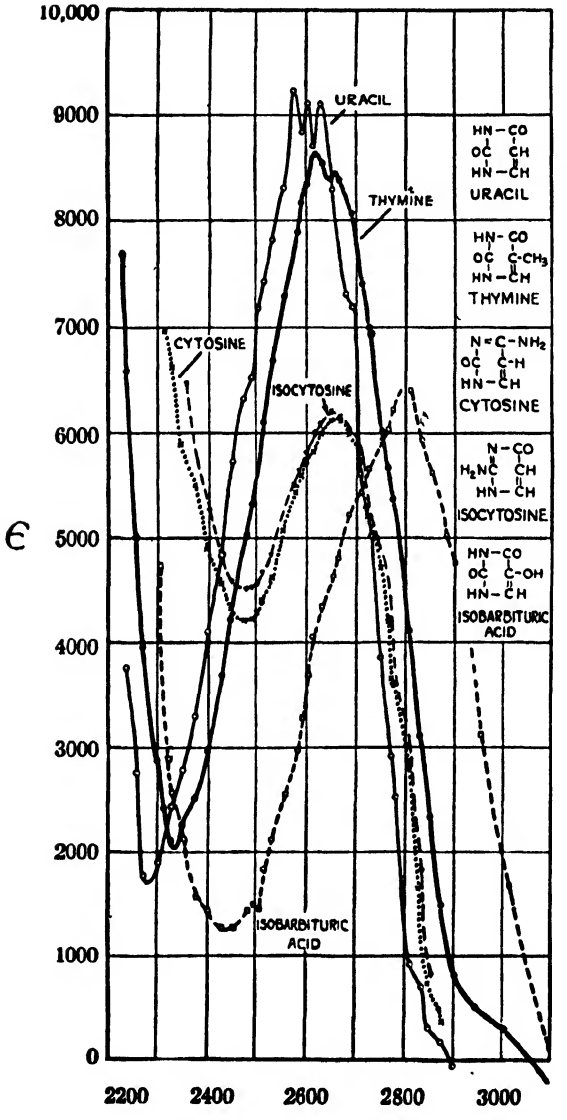
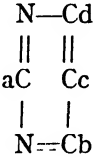


FIG. 66

After Heyroth and Loofbourow.

The same author (*ibid.*, 1932, **54**, 641) found that 6-phenyl uracil showed $\lambda_{max.}$ 286mμ whereas 1-alkyl 1,3-dialkyl,6-phenyluracils exhibited $\lambda_{max.}$ *ca* 275mμ, $\epsilon_{max.}$ being *ca* 10,000 throughout.

Heyroth and Loofbourow (*loc. cit.*) have also studied adenine, guanine and certain other purines. Selective absorption near 260mμ is again characteristic although $\epsilon_{max.}$ values tend to be higher. It is concluded that saturation of ethylenic linkages within the ring greatly decreases the absorption intensity, but that the presence of three double bonds has less effect than one or two



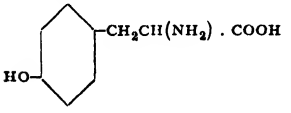
a	b	c	d	$\lambda_{max.}$ mμ	$\epsilon_{max.}$
CH_3	H	CH_3	NH_2	235	7,200
				265	6,800
CH_3	H	CH_2SO_3H	NH_2	248	10,000
				~265	7,500
CH_3	CH_3	H	NH_2	242	9,000
				~260	7,000
NH_2	CH_3	CH_3	NH_2	275	7,200
H	CH_3	NH_2	NH_2	292	10,800
H	NH_2	C_2H_5	NH_2	268	7,300
OH	CH_3	H	CH_3	215	9,000
				296	5,800
CH_3	CH_3	H	OH	228	6,100
				258	5,300
CH_3	H	CH_2SO_3H	OH	228	5,900
				275	5,900
NH_2	CH_3	H	CH_3	227	10,000
				287	4,000

CHAPTER IX

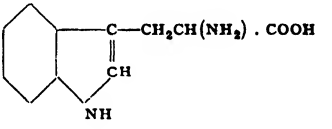
PROTEINS

The inter-relations of vitamins and co-enzymes and proteins make it necessary to summarise the position with regard to the absorption spectra of the proteins in general.

Out of all the amino-acids derived from proteins by means of acid or alkali or the action of proteolytic enzymes, only three are spectroscopically important, namely phenylalanine, tyrosine and tryptophane. Phenylalanine shows the feeble selective absorption of the C_6H_5 chromophore and this is normally masked by the intrinsically greater absorption of tyrosine



and tryptophane



Both these acids are classed among the essential amino acids. The indispensability of tryptophane was evident in 1915 (Osborne and Mendel) when it was shown that rats receiving zein (a vegetable protein deficient in tryptophane) as the proteinaceous part of a diet, rapidly lost weight, but as soon as tryptophane was added to the diet the loss of weight ceased. Tyrosine is also an essential amino acid, subject to the proviso that phenylalanine, if available in sufficient quantity, is capable of transformation into tyrosine *in vivo*. The obvious importance of the tryptophane and tyrosine content of proteins makes it necessary to study the application of spectrophotometry to the analysis of proteins. Many of the analyses reported in the literature must be erroneous; thus the tyrosine and tryptophane content of egg albumen has been given as:

tyrosine 1·77 to 5%
tryptophane " a trace " to 2·6%

(See Jordan Lloyd and Shore, *Chemistry of the Proteins*, 1938.)

Holiday (*Biochem. J.*, 1930, **24**, 619; 1936, **30**, 1795; 1938, **32**, 1166) has worked out a spectrophotometric method of considerable promise. Most proteins exhibit selective absorption in the region *ca* 280mμ, the exact position of $\lambda_{max.}$ depending on p*H*. Aliphatic amino-acids show weak continuous end absorption of rays $< 250m\mu$, and the selective absorption

of proteins is due to the aromatic amino-acids which they contain, and the intensity of absorption will clearly depend on the amounts present. Ross (*J. Biol. Chem.*, 1934, **104**, 531) determined phenylalanine in proteins free from both tryptophane and tyrosine, but it fell to Holiday to attempt the difficult task of estimating the proportions of these two acids.

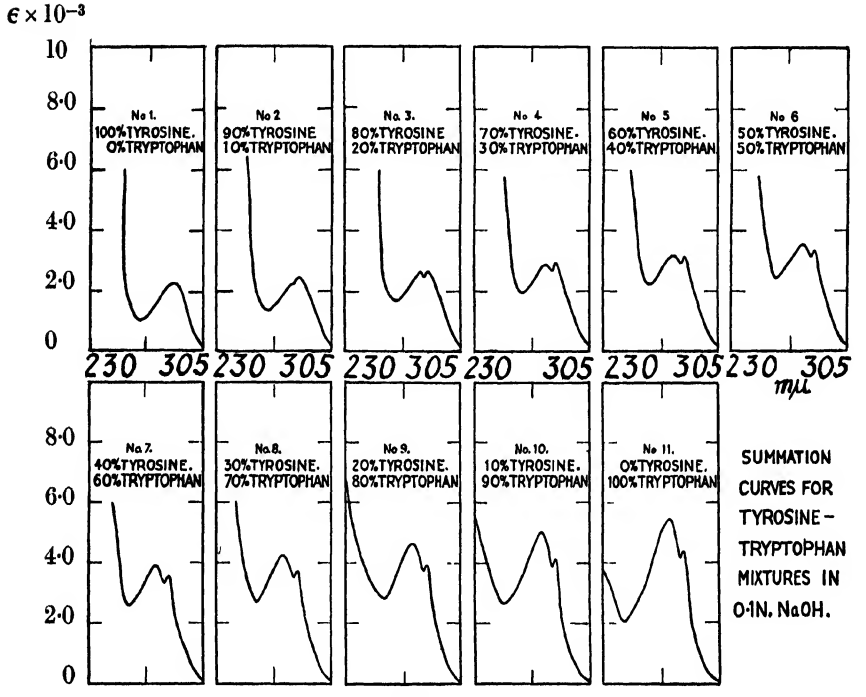


FIG. 67

Two conditions had first to be satisfied: (i) that the spectra of the two amino-acids should be unaffected by the fact that in proteins they occur linked to other amino-acids, (ii) that certain wave-lengths could be found at which both acids exhibit accurately measurable absorption, and at which *all* the absorption is due to these two constituents. Under such conditions Vierordt's method (*cf.* Twyman and Allsopp, *The Practice of Spectrophotometry*) for determining the concentrations of the components of a simple mixture from the individual spectra, can be applied with safety. Holiday chose the two wave-lengths 280mμ and 305mμ for solutions at $pH > 12$ and derived the equation

$$M(\text{tyrosine}) = 0{\cdot}99\, E_{305m\mu} - 0{\cdot}082\, E_{280m\mu} \times 10^{-3}$$

$$M(\text{tryptophane}) = 0{\cdot}0207\, E_{280m\mu} - 0{\cdot}280\, E_{305m\mu} \times 10^{-3}$$

where M represents molar concentration.

The results on sera, albumen, caseinogen, gelatin, gliadin and insulin showed that the method possessed great advantages on the score of ease and accuracy, but as compared with the best chemical data there was some tendency for the tyrosine figures to err on the high side and the tryptophane figures on the low side. The main source of error was irrelevant absorption due to pigments. Holiday applied a correction for this by determining the intensity of absorption of the protein in neutral or acid solution at 305mμ. Both tyrosine and tryptophane show negligible absorption at *ca* 301mμ over this p*H* range, so that any absorption extending beyond 310mμ in the direction of longer wave-lengths must be a measure of the concentration of pigment impurities. By subtracting the value of this irrelevant absorption from the $\bar{E}$ values at 305mμ and 280mμ observed in alkaline solution a truer measure of the relevant absorption can be obtained. In 1938 (*loc. cit.*) Holiday amplified this mode of correction.

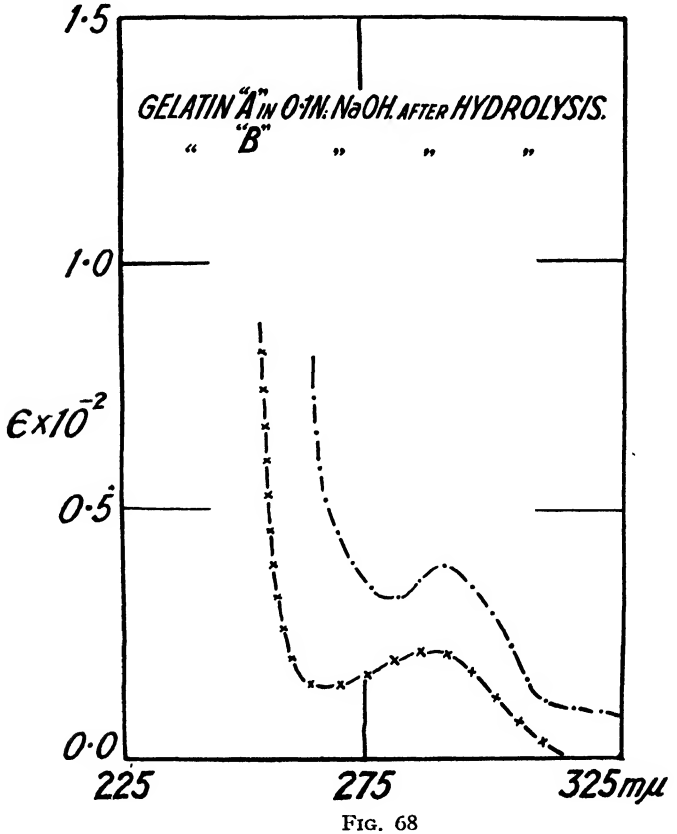


FIG. 68

Figs. 67 to 70 are based on unpublished work by Mr. T. W. Goodwin.

It is not always feasible, however, to make full use of his reasoning. Goodwin and the writer (unpublished work) have tried to refine the procedure by studying hydrolysates. Alkali hydrolysis of casein resulted in a coloured product and the spectroscopic data were unsatisfactory. Acid hydrolysis results in the complete destruction of tryptophane, but the degradation products are not themselves transparent in the region 270–305mμ. Tyrosine is not destroyed under these conditions. Recourse was then had to Lugg's method of hydrolysis (*Biochem. J.*, 1938, **32**, 775). The protein is hydrolysed with alkali-stannite in a sealed tube at 100° for 24–30 hours.

[Reagent: 5 g. $SnCl_2,2H_2O$ in 100 ml 5·5N NaOH (N_2 gas), 5 ml reagent + 1 g protein; air space not > 2 ml.]

The hydrolysate, after washing out three times with distilled water, was shaken three times for about one minute with three successive portions of zinc dust (0·2 g. per 10 ml. hydrolysate) and the product centrifuged. After pouring off the clear liquid and washing the residue with 0·1N. NaOH, the solution was twice extracted by means of ether. The aqueous layer was freed from dissolved ether by warming it in a current of nitrogen, and the solution diluted for spectrophotometric assay.

When applied to various proteins, such hydrolysates were found to show measurable absorption near 325mμ. Extrapolation of the pigment absorption is not difficult and quite satisfactory results are obtained.

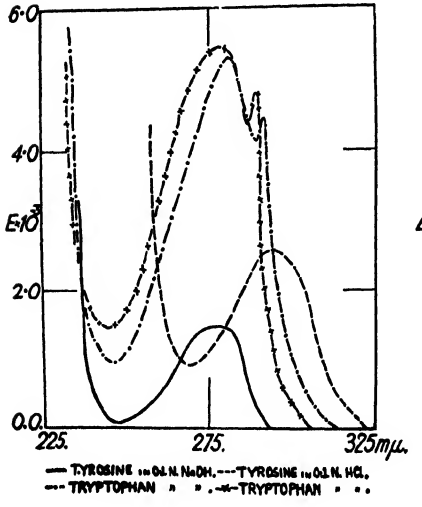


FIG. 69

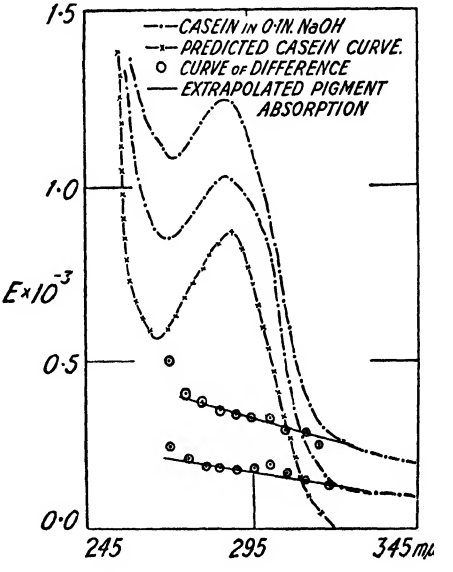


FIG. 70

The Lugg method of hydrolysis is easy to perform and a fairly accurate determination of tyrosine and tryptophane can be made spectrographically with very little material.

TABLE

in alkali

	λ_{max} mμ	$\epsilon_{max.} \times 10^{-2}$	$\lambda_{min.}$ mμ	$\epsilon_{min.} \times 10^{-2}$
Tyrosine	294	25·5	264	0·85
Tryptophane	281	50·3	246	9·5

in acid

	$\lambda_{max.}$ mμ	$\epsilon_{max.} \times 10^{-2}$	$\lambda_{min.}$ mμ	$\epsilon_{min.} \times 10^{-2}$
Tyrosine	277·5	14·96	246·5	0·05
Tryptophane	278	54·5	244	14·75

TABLE

Spectrophotometric Determination of Tyrosine and Tryptophane in Proteins.

LUGG'S ALKALI-STANNITE HYDROLYSIS.

	Without correction for irrelevant pigment absorption		after correction		
Protein	% Tyrosine	% Tryptophane	Tyrosine	Tryptophane	
Casein	7·8	1·2	5·1	Chemical	1·2	Chemical
				4·5		1·5
	11·9	0·7	5·2		1·2	
Zein	9·7	0·0	6·2		0·0	
				5·9		0·17
	11·1	0·21	5·5		0·3	
Gelatin A	1·2	0·0	0·28		0·47	
				0·001		0·00
Gelatin B	1·7	0·0	0·22		0·05	
Egg white	10·9	0·92	5·0		1·0	
				4·5		1·2
	10·4	0·57	5·2			

Provided of course that a protein can be obtained pure and there is no absorption in the region 310–325mμ, fairly accurate results can be obtained directly from the alkaline solutions.

There may well be a considerable future for the spectrographic method as a convenient tool for dealing with the proteins of physiological fluids.

CHAPTER X

ENZYMES AND COENZYMES

It is now impossible to review the state of knowledge concerning vitamins without some discussion of processes involving enzymes, and inasmuch as spectroscopy has played an important part in elucidating the complex interactions of different kinds of bio-catalysts, the topic of enzymes and coenzymes is specially relevant to the present work.

The ordinary concept of a protein is that of a compound of high molecular weight made up largely of amino-acid residues and responding to certain colour reactions and precipitation tests. Judged by such criteria many enzymes must fall into the same category. Most enzymes are non-dialysable, are labile to heat, and contain the same constituents as proteins and are of similarly high molecular weight. They are recognised by their catalytic rôle in specific chemical reactions. Coenzymes are also catalysts synthesised in the living cell, and although they are necessary to enzymic processes, they may (*a*) be separated from enzymes and studied *in vitro*, (*b*) be non-protein compounds more stable to heat and of ascertainable structure, and (*c*) they may be dialysable and of relatively low molecular weight. In many cases, then, enzymes are complexes of predominantly protein nature, and are made up of a bulky protein carrier (*apo*enzyme, " *Träger* ") to which is linked a material chemically different and detachable. This contains the prosthetic group concerned in the interaction between the substrate and the enzyme as a whole (holoenzyme). Although the coenzyme is the site of the process, the velocity of the reaction with the substrate and the specificity of the enzyme depend more on the nature of the particular protein acting as carrier. There are, however, a very large number of enzymes which have not been observed to possess a detachable prosthetic group, and although it would not be surprising if every enzyme were ultimately found to react at a specific active group, there is no evidence to exclude the normal amino-acids in polypeptide chains from functioning in such a way. Indeed the protein carrier *must* possess groups active in some degree in order to account for (*a*) the *apo*enzyme—*co*enzyme attachment, and for (*b*) the specificity of enzyme-substrate interactions. The same coenzyme, *e.g.* cozymase, in association with different " carriers " may form a variety of enzymes

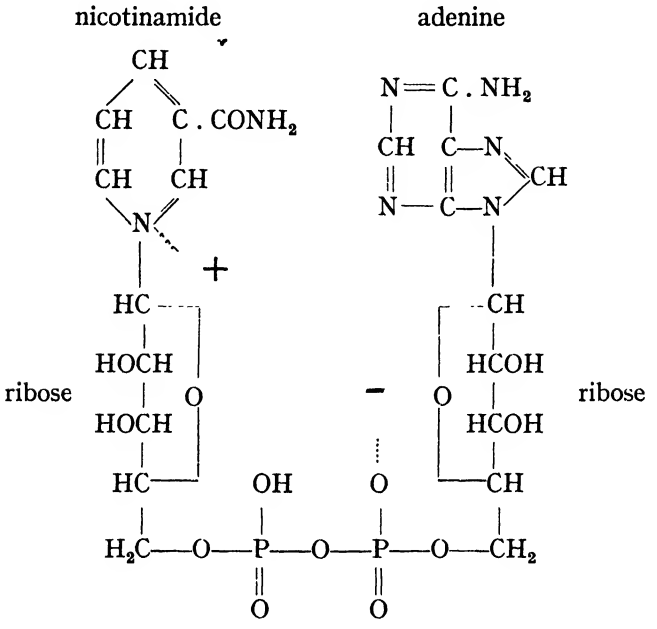
resembling each other in the general nature of the processes which are catalysed, but differing in substrate-specificity. Thus to give another instance, catalase and peroxidase probably require the same iron porphyrin coenzyme, but the carrier is different. In the carrier—coenzyme complex it is therefore fair to regard both components as highly specific to the final enzyme—substrate process. Whether the forces binding coenzymes to carriers are those of ordinary chemical linkages or those concerned in adsorption is an open question.

The term coenzyme was first used in 1897 for Ca^{++} and Mn^{++} as essential inorganic constituents, but the first example of a true coenzyme emerged from the work of Harden and Young (1904) who obtained, from boiled yeast juice, a heat-stable dialysate which greatly increased the fermentation of cell-free extracts. Since the enzyme was already known as zymase the coenzyme was designated cozymase. Meyerhof (1918) obtained the same substance from milk and many animal organs, and showed that it is conveniently obtained from frog muscle by extracting with boiling water. The boiled juice ("Kochsaft") increased the respiration of minced muscle. The chemistry of fermentation and of muscle action were thus found to be related through a common catalyst. Szent-Györgyi later found that cozymase also forms part of the lactic acid—dehydrogenase system.

Warburg, Christian and Griese (*Biochem. Z.*, 1935, **282**, 157) following earlier work (*ibid.*, 1932, **254**, 438; 1935, **279**, 143), succeeded in oxidising hexose monophosphate *in vitro* by oxygen, and the interpretation they advanced contributed greatly towards elucidating the complex mechanism of the process. The system consisted of a dehydrogenase (from yeast), a yellow enzyme (see p. 94), a coenzyme from red blood cells, oxygen, and the hexose monophosphate substrate. In the presence of the dehydrogenase, the coenzyme was reduced by accepting 2H from the hexose monophosphate, the reduced coenzyme transferred 2H to the yellow enzyme, which was in turn reoxidised by O_2 yielding H_2O_2. The coenzyme was isolated and found to be a dinucleotide made up of adenine (1), nicotinamide (1), pentose (2), and phosphoric acid (3), the numbers in brackets indicating molecular proportions. The reversible oxidation and reduction of the coenzyme occurs in the nicotinamide residue (see p. 189). A little later Warburg and Christian (*ibid.*, 1936, **285**, 156; **287**, 291) found that cozymase also contained nicotinamide and functioned similarly in respect of reversible oxidation and reduction.

In the field of enzyme chemistry the problem of nomenclature causes much difficulty. Thus coenzyme I, cozymase, co-dehydrase (Euler), yeast coenzyme, lactic acid coenzyme are all synonymous with the

diphosphopyridine nucleotide (DPN) visualised (Euler and Schlenk, *Z. physiol. Chem.*, 1936, **246**, 64) as:



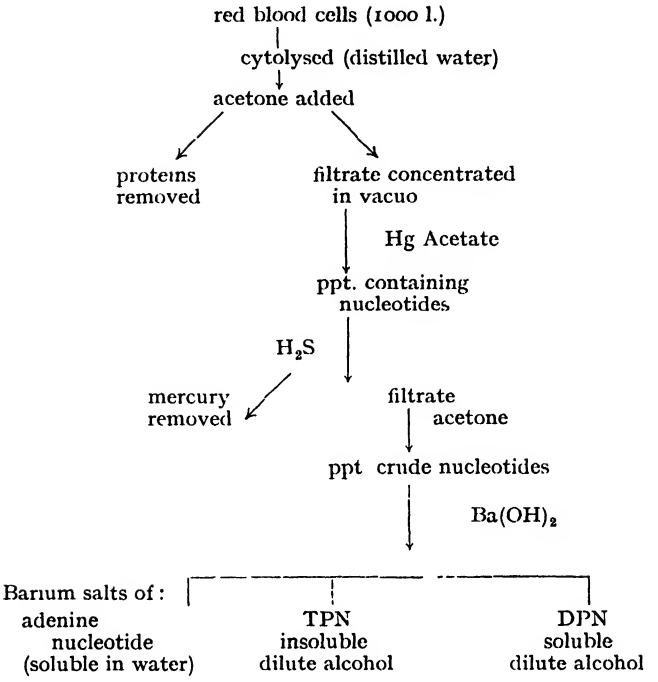
The hydrogen-transporting enzyme of red blood cells is variously known as coenzyme II, co-ferment, codehydrase II and hexose monophosphate coenzyme, and is the corresponding tri-phosphopyridine nucleotide (TPN). It is regarded by Warburg as a ubiquitous cell constituent. Treatment of DPN with $POCl_3$ results in TPN (Schlenk, *Naturwiss.*, 1937, **25**, 668).

The coenzymes may be obtained from various sources by methods worked out by Warburg, Euler, Keilin and their associates, and others (*cf.* Ohlmeyer, *Biochem. Z.*, 1938, **297**, 66), but the principle is well illustrated by a procedure due to Warburg. (*See opposite page.*)

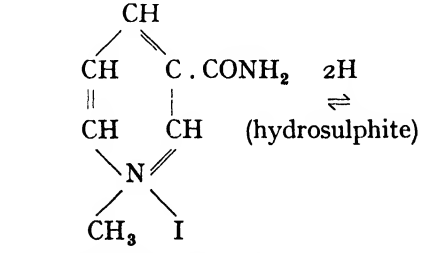
Further purification yields the three nucleotides as individual substances.

The pyridine nucleotides are acidic, water-soluble, dialysable substances which readily form salts (with heavy metals), the solubilities of which in water or dilute alcohol can be utilised in fractionation. Chromatographic analysis (see p. 59) is useful in this field because TPN is more firmly adsorbed on alumina than DPN, and phosphoric acid can be used for elution.

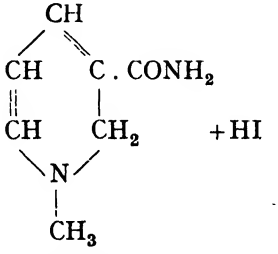
WARBURG'S PROCEDURE.



Both coenzymes I and II form dihydrides Co . 2H on reduction with sodium hydrosulphite. In alkaline solution the reaction mixture displays a transient yellow colour which also occurs in the reduction of quite simple pyridine derivatives. Thus Karrer and Benz (*Helv. Chim. Acta*, 1936, **19**, 1028) studied the process:



nicotinamide methiodide



reduced form

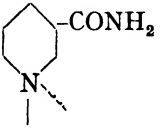
and observed that the dihydride is strongly fluorescent (under ultra-violet irradiation) and exhibits an absorption band $\lambda_{max.}$ *ca* 340mμ, not

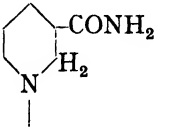
shown by the oxidised form. Both coenzymes on reduction (in an enzyme system or by hydrosulphite) exhibit a similar fluorescence and a strictly analogous change in absorption spectrum. On shaking with methylene blue or flavoprotein (yellow enzyme preparation) and oxygen they readily revert to the higher state of oxidation.

The reaction which occurs when coenzyme I facilitates the oxidation of hexose monophosphate in the presence of the dehydrogenase may thus be written

$$C_6H_{11}O_6 \cdot H_2PO_3 + CoI + H_2O \longrightarrow C_6H_{11}O_7 \cdot H_2PO_3 + CoI \cdot 2H + H^+$$

(DPN) — phosphohexonic acid





If flavoprotein is added to the same system and air excluded, the result is

$$Co \cdot 2H + \text{flavoprotein} \rightarrow Co + \text{flavoprotein} \cdot 2H$$

the hydrogen from the reduced coenzyme being transferred to the alloxazine part of the flavoprotein and the coenzyme regenerated so as to participate afresh in hydrogen transfer. Immediately oxygen is introduced the reduced flavoprotein is oxidised with quantitative liberation of H_2O_2

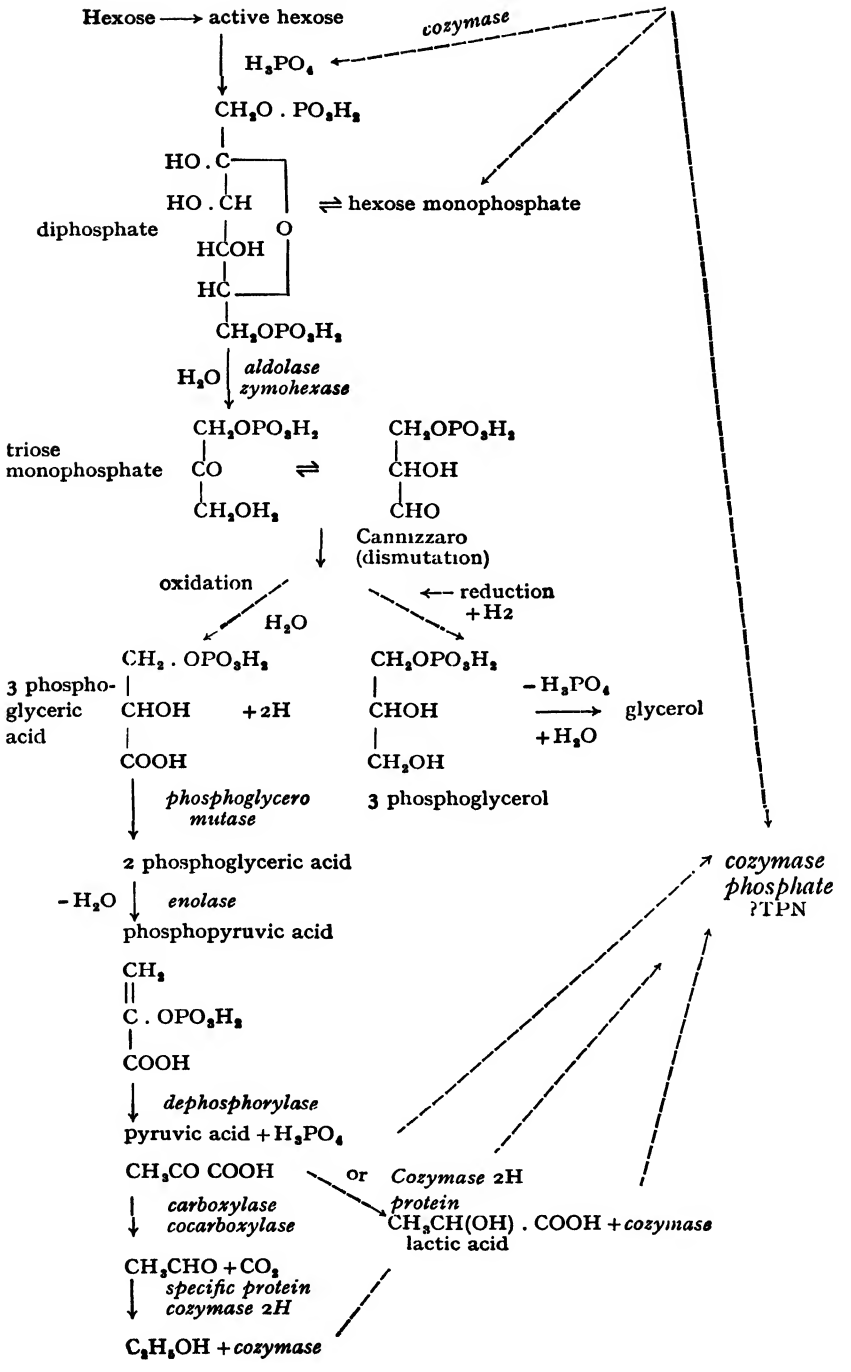
$$\text{flavoprotein} \cdot 2H + O_2 \rightarrow \text{flavoprotein (regenerated)} + H_2O_2$$

The entire process is catalytic since neither coenzyme nor yellow enzyme is used up.

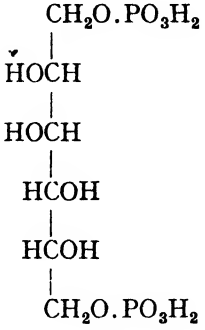
In the living cell, flavoprotein may be replaced by the cytochromes and cytochrome oxidase (*cf.* many papers by Keilin and his colleagues), and by diaphorase and other intermediaries.

Only a very imperfect account of recent work would be given if stress were not laid on the consecutive nature of enzymic processes, thus the phosphohexonic acid resultant is itself a substrate for further oxidation. In the presence of coenzyme II (TPN) yeast protein and flavoprotein, phosphohexonic acid absorbs $3O_2$ and yields $3CO_2$.

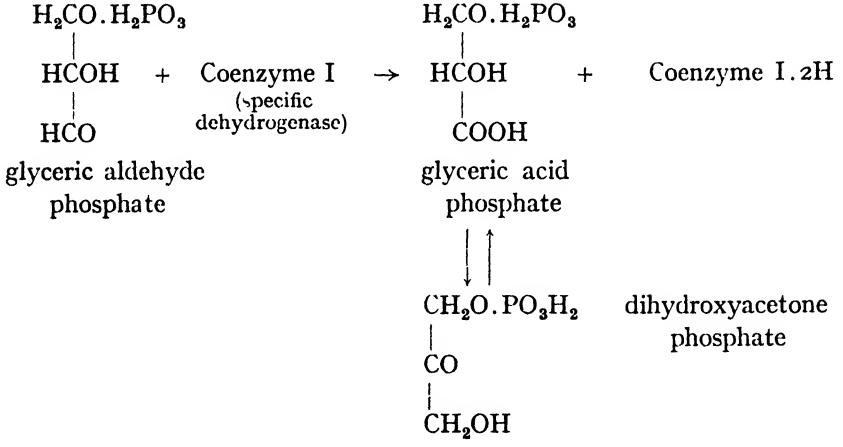
The fermentable monosaccharides (glucose, fructose, mannose), in the active enol form, unite with phosphoric acid to form a common hexose


Hexose → active hexose
cozymase
H_3PO_4
$CH_2O . PO_3H_2$
HO . C
HO . CH
⇌ hexose monophosphate
diphosphate
O
HCOH
HC
$CH_2OPO_3H_2$
H_2O
aldolase
zymohexase
$CH_2OPO_3H_2$
$CH_2OPO_3H_2$
triose monophosphate
CO
⇌
CHOH
CH_2OH
CHO
Cannizzaro (dismutation)
oxidation
reduction
+H2
H_2O
$CH_2 . OPO_3H_2$
3 phospho-glyceric acid
CHOH
+2H
COOH
$CH_2OPO_3H_2$
CHOH
CH_2OH
$-H_3PO_4$
$+H_2O$
glycerol
phosphoglycero mutase
3 phosphoglycerol
2 phosphoglyceric acid
$-H_2O$
enolase
phosphopyruvic acid
CH_2
C . OPO_3H_2
COOH
dephosphorylase
pyruvic acid + H_3PO_4
cozymase phosphate
?TPN
CH_3CO COOH
or
Cozymase 2H
protein
carboxylase
cocarboxylase
$CH_3CH(OH)$. COOH + cozymase
lactic acid
$CH_3CHO + CO_2$
specific protein
cozymase 2H
C_2H_5OH + cozymase

diphosphate, and it is quite likely that cozymase itself enters into phosphate transfer in conjunction with phosphatases. It certainly plays a



part in the subsequent fate of hexose diphosphate, whether it is dehydrated or fermented. In fermentation, it undergoes fission and the resulting triose phosphate loses 2H, *e.g.*



It will be noticed that the conversion of pyruvic acid to acetaldehyde involves a decarboxylating enzyme, carboxylase. Auhagen (*Z. physiol. Chem.*, 1932, **204**, 149) discovered that the enzyme possessed a detachable prosthetic component which he called cocarboxylase. The system carboxylase—cocarboxylase converts α-ketoacids to aldehydes:

$$R.CO.COOH \rightarrow R.CHO + CO_2$$

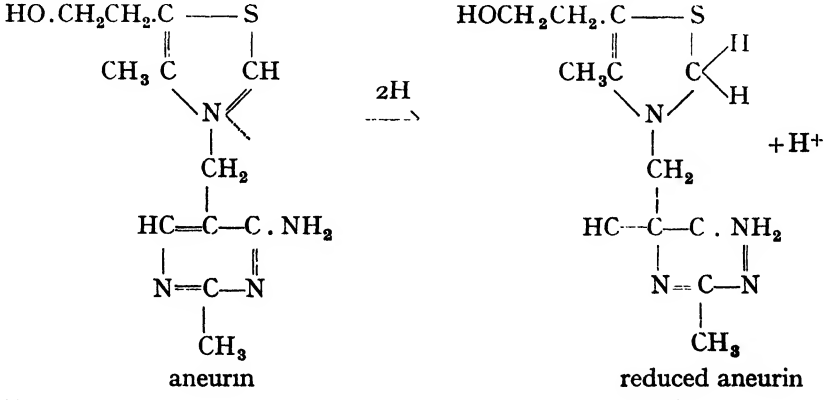
Simola (*Biochem. Z.*, 1932, **254**, 229) found that the brain and liver of rats suffering from avitaminosis B_1 contained abnormally small amounts of cocarboxylase. It was not, however, for some time that the nature of the connexion was made clear. Lohman and Schuster (*Biochem. Z.*, 1937, **294**, 188) isolated the coenzyme and established that it consisted

of aneurin (vitamin B_1) combined with pyrophosphoric acid. The name cocarboxylase is widely retained, although aneurin (often called thiamin in America) pyrophosphate is accurate.

Cocarboxylase occurs in yeast, liver and kidney and in many vegetables. Brewer's yeast is a good source, yielding perhaps 75 p.p.m. of cocarboxylase hydrochloride. The coenzyme is formed from aneurin (Kinnersley and Peters, *J. Soc. Chem. Ind.*, 1937, **56**, 934) by enzymic processes and by growing yeast. It may be prepared *in vitro* from aneurin hydrochloride and a mixture of sodium pyrophosphate and orthophosphoric acid (Tauber, *J. Amer. Chem. Soc.*, 1938, **60**, 730, 2263).

The thiochrome reaction (p. 152) may be used to determine aneurin and cocarboxylase in tissue (Westenbrink and Jansen, *Acta. brev. Neerl.*, 1938, **8**, 119). Oxidation [$K_3Fe(CN)_6$] gives free thiochrome and thiochrome pyrophosphate, and if the alkaline aqueous solution is shaken with *iso*butyl alcohol, only the former is extracted. On exposure to ultraviolet light both solutions fluoresce strongly, and from the intensities it is possible to determine concentrations (Westenbrink and Goudsmit, *Nature*, 1938, **142**, 150; Hennessy and Cerecedo, *J. Amer. Chem. Soc.*, 1939, **61**, 179). Cocarboxylase shows the aneurin absorption spectrum ($\lambda\lambda_{max.}$ 245, 260mμ).

The constitution of cocarboxylase makes clear, at any rate in respect of one function, the mechanism by which vitamin B_1 is utilised. In vitamin B_1-deficient animals, the pyruvic acid content both of brain and blood rises, but becomes normal when aneurin is administered. The simple decarboxylation of pyruvic acid occurring in yeast is apparently not reproduced in animal tissues, and it is possible that cocarboxylase acts on another protein to form a pyruvic acid dehydrogenase. This was shown by the work of Peters (*Biochem. J.*, 1937, **31**, 2240) and Lipmann. An investigation on the hydrogenation of aneurin and other thiazoles (Lipmann and Perlmann, *J. Amer. Chem. Soc.*, 1938, **60**, 2574) shows that the catalytic action of thiazoles is akin to that of pyridinium compounds:



aneurin reduced aneurin

The hydrogen is again added on at the double bond adjoining the quaternary nitrogen. In the reaction between hydrosulphite and aneurin

$$Na_2S_2O_4 + R + 2NaHCO_3 \rightarrow 2Na_2SO_3 + R \cdot 2H + 2\ CO_2$$

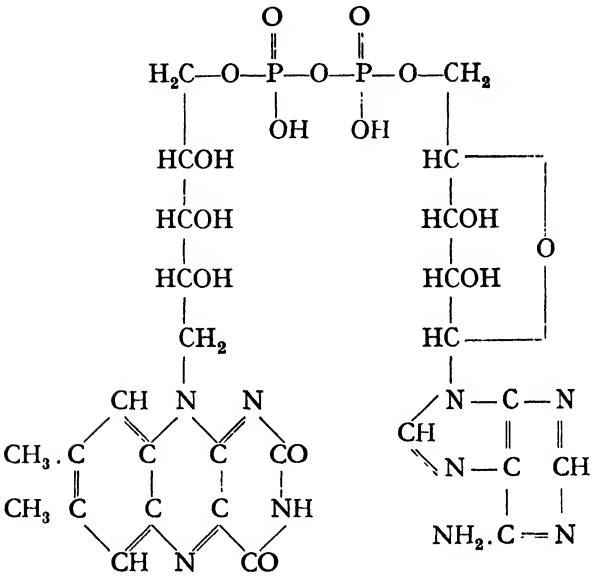
the process may be followed by Warburg's manometric method. The idea that the thiazole group acts as a hydrogen " transporter " is supported by the appearance of a transient greenish-yellow colour when hydrosulphite is added to neutral solutions of aneurin because similar transient colours are seen with all quaternary thiazoles on reduction by means of hydrosulphite. The colour is probably due to an intermediate of the " semi-quinone " type.

Reference has already been made to " flavoprotein ". Warburg's classical work on the yeast dehydrogenase of hexose monophosphate led to the isolation of his " yellow enzyme ". Following on the isolation of riboflavin (lactoflavin, ovoflavin, hepatoflavin, etc.), Theorell (*Biochem. Z.*, 1935, **278**, 263) established that the yellow enzyme contained riboflavin phosphate as the prosthetic group. By dialysing a solution of the enzyme against 0·02N HCl for three days he was able to separate the protein without denaturation from the yellow component. Recombination to give an active enzyme occurred readily, but riboflavin itself (as distinct from the phosphate) failed to form an active product with the specific protein.

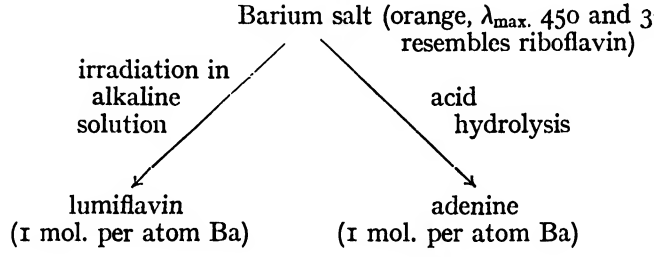
The rôle of riboflavin in coenzymes is, however, much more complicated than would appear from the foregoing. Krebs (*Z. physiol. Chem.*, 1933, **217**, 191) recognised the existence of an enzyme (amino-acid oxidase) which could catalyse the following reaction :

$$R \cdot CHNH_2 \cdot COOH + O \rightarrow R \cdot CO \cdot COOH + NH_3$$

The following steps were important in the further study of this deaminase (amino-acid oxidase) : (*a*) a heat stable factor was shown to be part of the enzyme system (Das, *Biochem. J.*, 1936, **30**, 1080, 1617) ; (*b*) a coenzyme component was separated by dialysis (Warburg and Christian, *Biochem. Z.*, 1938, **295**, 261) ; (*c*) a parallelism was established between enzymic activity and selective absorption (flavin content) in different coenzyme preparations (Straub, *Nature*, 1938, **141**, 603) ; (*d*) Warburg and Christian (*Biochem. Z.*, 1938, **298**, 150) established unequivocally the constitution of the coenzyme as an alloxazine-adenine-dinucleotide, possessing in all probability the following structure :



Yeast, kidney and liver are among the best sources, but the preparation is tedious and yields of pure material are scanty. Perhaps the crux of the whole problem was reached when the barium derivative of the dinucleotide was obtained pure (Warburg and Christian, *Naturwiss.*, 1938, **26**, 201):



The spectrum of the coenzyme is not very different from a summation of the riboflavin and adenine curves.

The *modus operandi* is :

aminoacid *minus* 2H → reduced coenzyme
(2H adds on at the alloxazine ring)

reduced coenzyme + O_2 → coenzyme

According to Warburg, 2880 molecules of alanine are oxidised per minute in the system for every molecule of the dinucleotide coenzyme present. When it is united with a different protein component, the coenzyme can act as intermediary in the oxidation of xanthine and reduced DPN or TPN, the particular protein determining the specificity (but see Corran and Green, *Biochem. J.*, 1938, **32**, 2231, on a new flavoprotein from milk). Perhaps the most remarkable flavoprotein is Straub's diaphorase from heart muscle (*Nature*, 1939, **143**, 76; Straub, Corran and Green, *ibid.*, **143**, 119). One molecule of this flavoprotein (the prosthetic group of which is also an alloxazine-adenine-dinucleotide) catalyses the oxidation of 8500 molecules of reduced cozymase per minute at 38°. This is the highest turnover so far recorded in this field.

Three types of coenzymes have now been considered in which a vitamin-like body (nicotinamide, aneurin, riboflavin) plays a dominant part. This raises an interesting question concerning adenine. With riboflavin mononucleotide and the flavin-adenine-dinucleotide as coenzymes, adenylic acid—or rather adenyl pyrophosphate—must also be accepted as a coenzyme, which Euler, quite legitimately, calls cophosphorylase. It provides a mechanism concerned with anaerobic glycolysis and aerobic respiration in muscle.

$$\text{Adenyl pyrophosphate} + \text{hexose} \xrightarrow{\text{phosphate transfer}} \text{hexose phosphate} + \text{adenylic acid}$$

$$\text{phosphopyruvic acid} + \text{adenylic acid} \rightarrow \text{adenyl pyrophosphate} + \text{pyruvic acid}$$

i.e. adenylic acid and adenyl pyrophosphate act as coenzymes.

[in addition: adenyl pyrophosphate → adenylic acid may occur through creatin]

It is interesting to speculate whether *adenine* may not behave in some senses as a vitamin.

Perhaps the most finished piece of work in enzyme mechanisms is that of Negelein and Wulff (*Biochem. Z.*, 1937, **293**, 351). The reduction of acetaldehyde to alcohol follows the scheme:

$$DPN(H_2) + CH_3CHO \xrightarrow{\text{protein}} DPN + C_2H_5OH$$

The enzyme is a specific proteid with cozymase as the prosthetic group and the conversion of nicotinamide to dihydro nictotinamide approximates to the actual mechanism. The protein part of the enzyme was obtained (from bottom yeast) in crystalline form, it shows the normal protein spectrum ($\lambda_{max.}$ 280mμ) and appears to be practically pure (P, 2×10^5g. protein per g. atom P; Fe, 2×10^6 g. protein per g. atom Fe). The reduced DPN combines with the protein about three times as efficiently

as DPN itself. By very skilful work Negelein and Wulff were able to follow the kinetics of the enzyme reaction in detail using the 340mμ absorption band for determining the concentration of reduced DPN (photoelectric technique). The whole process has now been worked out, and only the nature and structure of the protein remains to be elucidated (*cf.* Green and Dewan on the oxidation and reduction of coenzyme I ; *Biochem. J.*, 1937, **31**, 1070).

Virus problems

In recent years, proteins specific to plants infected with disease (*e.g.* tobacco mosaic virus) have been obtained crystalline. It appears that these proteins are the viruses themselves. They are all nucleoproteins but they differ among themselves, and from the point of view of this book the important fact is that they differ in respect of nucleic acid content. Little spectroscopic work has been done in this field, but there is some chance that the nucleic acid content may be ascertainable by analysis of the spectra in such a way as to separate the contributions due to the amino-acids tyrosine and tryptophane, and the purine bases (*cf. Proc. Roy. Soc.*, 1938, **B125**, 291–310 ; Bawden and Pirie, *Nature*, 1938, **141**, 513 ; **142**, 842).

Crystalline Enzymes

One of the objectives of enzyme research is to obtain crystalline specimens. This aim has been reached in about a dozen cases, *e.g.* urease (Sumner), catalase (Sumner and Dounce), pepsin (Northrop), pepsinogen (Herriot and Northrop), trypsin and trypsinogen, chymotrypsin, chymotrypsinogen (Kunitz and Northrop), carboxypeptidase (Anson), etc. Crystalline preparations are not necessarily pure substances, they may contain inert protein and non-protein nitrogen from protein degradation products. According to Northrop and Herriott (*Ann. Rev. Biochemistry*, 1938, 37), the only criterion of purity which has not so far been seriously questioned depends on solubility. The available evidence suggests that a large number of crystalline enzymes are spectroscopically indistinguishable from ordinary proteins.

A number of natural catalysts entering into cellular oxidations are now known to be proteins in which the prosthetic group is an iron-containing porphyrinic compound belonging to the general class of haematins. Warburg's famous " Atmungsferment " represents the sum total of the iron compounds acting as catalysts in the cell. Catalase (Zeile and Hellström, *Z. physiol. Chem.*, 1930, **192**, 171 ; 1931, **195**, 39) was obtained fairly pure some years ago and for the first time a direct proportionality between catalytic activity and the intensity of light

absorption due to porphyrin-bound iron was established. Kuhn, Hand and Florkin (*ibid.*, 1931, **201**, 255) claimed that peroxidase was similar in this respect. The evidence was, however, inadequate but later work by Keilin and Mann (*Proc. Roy. Soc.*, 1937, **B122**, 119) established clearly that peroxidase is in fact a compound of protohaematin with native protein. The same haematin nucleus attached to three distinct native proteins gives rise to methaemoglobin, catalase and the peroxidase of horse radish.

The absolute activities of peroxidase, catalase and " Atmungsferment " are all of the same order, namely 10^5 mols. of H_2O_2 or O_2 are used up per mol. of enzyme. The activity depends (*a*), on the manner in which the haematin group is adsorbed, and (*b*), on the mode of linkage of the iron atoms, thus :

1 gram atom catalase iron decomposes 6×10^4 to 2×10^5 mol. H_2O_2 at O°.
1 ,, ,, haemin iron ,, 0·01 mol. H_2O_2 at O°.
1 ,, ,, Fe^{++} or Fe^{+++} ,, 0·00001 mol. H_2O_2 at O°.

In order to explain the current view concerning the mechanism of intracellular oxidation it is necessary to deal with Keilin's classical work on cytochrome. In 1886–1890 MacMunn (*Phil. Trans.*, 1886, **177**, 267 ; *J. Physiol.*, 1887, **8**, 57 ; *Z. physiol. Chem.*, 1889, **13**, 309) described a respiratory pigment histohaematin (myohaematin) present in many tissues, and particularly muscle. The pigment could readily be reduced, and although in the oxidised form it showed no characteristic selective absorption, in the reduced state it showed four distinct absorption bands :

615–593, 567·5–561, 554·5–546, 532–511mμ.

MacMunn concluded that the pigment differed from haemoglobin and its known derivatives. Unfortunately the work attracted adverse comment, particularly from Hoppe-Seyler, and it was gradually almost forgotten.

Keilin (*Proc. Roy. Soc.*, 1925, **B98**, 312), in the first of a series of papers, established not only the real existence of MacMunn's pigment, but showed it to be of greater significance than its discoverer could ever have realised. Keilin used the term cytochrome (= cellular pigment) for the product, and, by using the microspectroscopic ocular of Zeiss and the Hartridge reversion spectroscope, was able to measure its absorption spectrum. Maxima at 603·5, 564·5, 549 and 519mμ could be seen merely by slightly wetting bakers' yeast. The same bands could be seen by direct examination of the almost transparent thoracic muscles of bees. Oxidised cytochrome showed no distinct bands, but, on reduction ($Na_2S_2O_4$), the characteristic absorption reappeared. Cytochrome is an intracellular respiratory cata-

lyst common to animals, bacteria, yeast and higher plants. In the reduced state the spectrum shows maxima at 604·6, 566·5, 550·2, 521mμ (the latter containing three subsidiary maxima) but the relative intensities are not constant, and it is evident the cytochrome is not a single substance. It is in fact made up of at least three cytochromes (*a*, *b* and *c*) which differ in important respects although they have much in common. There are three haematin compounds in the cytochrome of the cells of aerobic organisms and an additional unbound haematin. Cytochrome is easily oxidised by air and reduced by the normal activity of cells or by a chemical reducer, although components *a* and *c* are not autoxidisable whilst *b* is. The oxidation of cytochrome is inhibited by $N/_{10,000}$ KCN or by sodium pyrophosphate.

Keilin also found that yeast and muscle contain an insoluble thermostable oxidase (recognised experimentally by its action on indophenol). This material is inactivated by traces of KCN and by carbon monoxide in the dark. The oxidase-CO compound decomposes on illumination with regeneration of the active oxidase. The indophenol- or cytochrome-oxidase, as it was later called, plays a decisive rôle in cell respiration, and in particular in the oxidation of cytochromes *a* and *c*. Keilin enunciated the view that cytochrome in living cells is reduced by a variety of organic metabolites, which become hydrogen donators under the action of dehydrogenases. The cytochrome thus reduced is acted upon by the (indophenol) oxidase and regenerated in the oxidised form.

The component *c* was obtained (*Proc. Roy. Soc.*, 1930, **B106**, 418), in a concentrated form, as a transparent red solution from bakers' yeast. It was not autoxidisable and failed to catalyse the oxidation of cysteine. Cytochrome oxidase may be obtained from heart muscle, and it also fails to oxidise cysteine, but when cytochrome *c* and the oxidase are both present, cysteine is very rapidly oxidised. The activity of the mixture is lost at 70° and is inhibited by 0·001N KCN. In the dark, high concentrations of carbon monoxide result in much diminution of activity but illumination brings about recovery. Dixon, Hill and Keilin (*ibid.*, 1931, **B109**, 29) determined the absorption spectrum of cytochrome *c* quantitively. Zeile and Reuter (*Z. physiol. Chem.*, 1933, **221**, 101) improved on Keilin's method and Theorell (*Biochem. Z.*, 1935, **279**, 463; 1936, **285**, 207) obtained pure cytochrome *c* from ox heart. It contained 0·34% of iron. Keilin and Hartree (*Proc. Roy. Soc.*, 1937, **B122**, 298) obtained far better yields of the pure substance by extracting freshly minced heart muscle with trichloracetic acid, followed by a fractional precipitation with $(NH_4)_2\ SO_4$ and $CCl_3 . COOH$. The absorption spectrum of reduced cytochrome *c* is shown in Fig. 72.

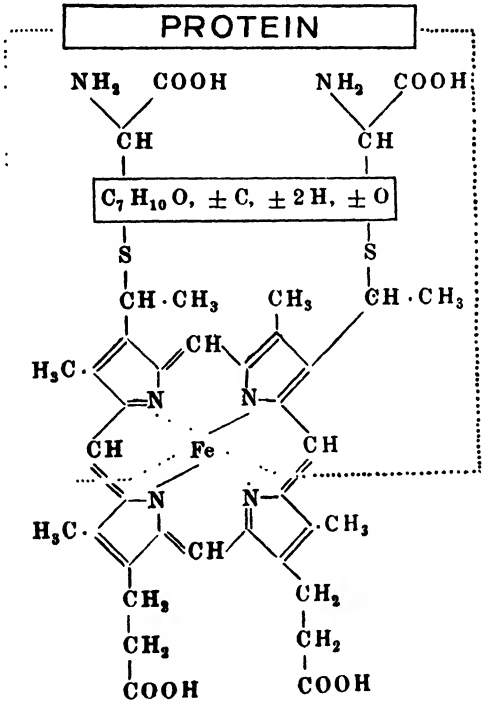
The concentration c of any solution may be determined spectrophotometrically from the relation :

$$c = \log_{10} I_0/I \times 1{\cdot}86 \times 10^{-8}$$

(c = concentration of cytochrome c in gram atoms iron per c.c.
d = thickness in cms.)

Theorell (*Biochem. Z.*, 1938, **298**, 202) has studied the chemical structure of pure cytochrome c.

The formula illustrates his ideas :



A porphyrin c is obtained by the action of acid on cytochrome c and if fission is carried out by hydrogen bromide in acetic acid two products are obtained, one a haematoporphyrin and the other a complex material with two sulphhydryl groups and two α-amino acid groups. The latter were evidently originally combined with the protein by peptide linkage. The prosthetic iron-porphyrin compound is therefore attached to the protein through S groups.

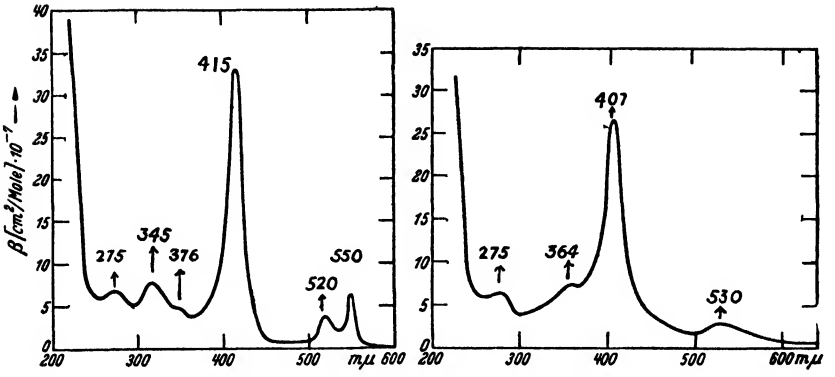


FIG. 71
Cytochrome *c*
After Theorell

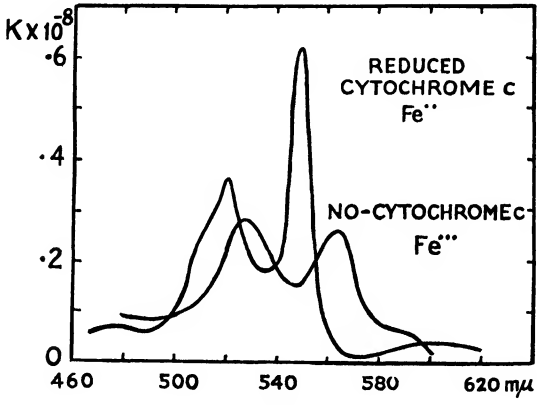


FIG. 72

In a paper on cytochrome oxidase, Keilin and Hartree (*Proc. Roy. Soc.*, 1938, **B125**, 171) remark that " although a great deal of work has been devoted to the study of this enzyme, the literature of the subject still contains a great bulk of inaccurate and controversial statements and faulty interpretations of observed facts ". In what follows, the writer is mainly utilising a very recent paper by the same authors (*Proc. Roy. Soc.*, 1939, **B127**, 167). Cytochrome oxidase is best obtained from washed minced heart muscle of mammals. The preparation is a very finely divided suspension of muscle tissue which shows the complete cytochrome spectrum (after reduction with $Na_2S_2O_4$) in a 2 mm. layer. Relatively to components *b* and *c* the concentration of cytochrome *a* is specially high. The fresh preparation does not absorb O_2 and the cytochrome is present in the oxidised state. Reduced cytochrome *c* when added to the suspension is rapidly oxidised even when only traces of oxygen are present. The

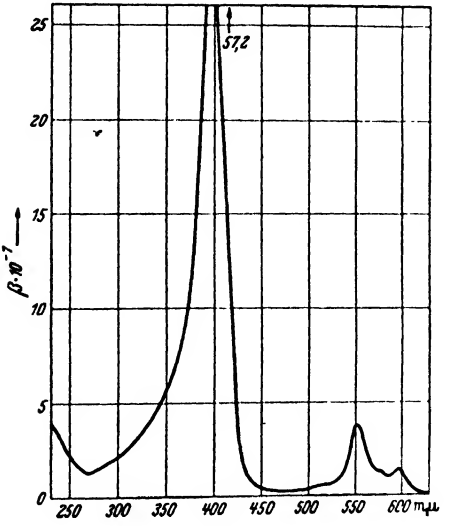


FIG. 73
Absolute absorption of porphyrin *c* in N. HCl.
After Theorell.

oxidase is not in solution. Addition of cytochrome *c* (10^{-4} - $10^{-5}M$) to heart muscle preparation greatly accelerates the catalytic oxidation of *p*-phenylene diamine, hydroquinone, *p*-aminophenol and similar substances which reduce cytochrome *c*. The " indophenol " oxidase seems to work *only* through the co-operation of cytochrome and should therefore be known as cytochrome oxidase (*cf.* also Stotz, Sidwell and Hogness, *J. Biol. Chem.*, 1938, **124**, 733).

The heart muscle preparation contains no haemoglobin but in it there are several enzymes and carriers which are either insoluble or strongly bound

Description of Plate VII. *facing p.* 206.

Diagrammatic figure of reconstructed absorption spectra of reduced and oxidised cytochrome in heart-muscle preparation, untreated and treated with different respiratory inhibitors. Bands of each spectrum are represented as seen in three different depths of preparation ; their ratio, according to bands, being approximately as follows : 8 for α and β bands ; 2·5 for $a\gamma$ and $a_3\gamma$; 1·5 for $b\gamma$ and $c\gamma$. Band $c\gamma$ is seen more distinctly because deficiency of this component in this preparation is compensated by addition of soluble *c*. Notation of bands given above and below diagram refers only to spectra I and VII respectively. Spectrum II shows $a_3'CO$ α-band at 590mμ, while band $a_3CO\gamma$ appears at 432mμ, where it overlaps and intensifies band $\beta\gamma$. Band $\alpha\gamma$ is seen at 452mμ. In spectrum III bands α and γ of a_3 are invisible owing to its oxidation and combination with inhibitors. Spectra IV, V and VI are clearly explained in the figure. Spectrum VII, representing oxidised cytochrome reconstructed from different depths of preparation, shows remains of reduced α-band, two diffuse parahaematin bands ($p\alpha$ and $p\beta$) of compounds *b* and *c*, and two diffuse bands (*f*) of a flavoprotein compound, which become hardly perceptible in preparations showing reduced cytochrome.

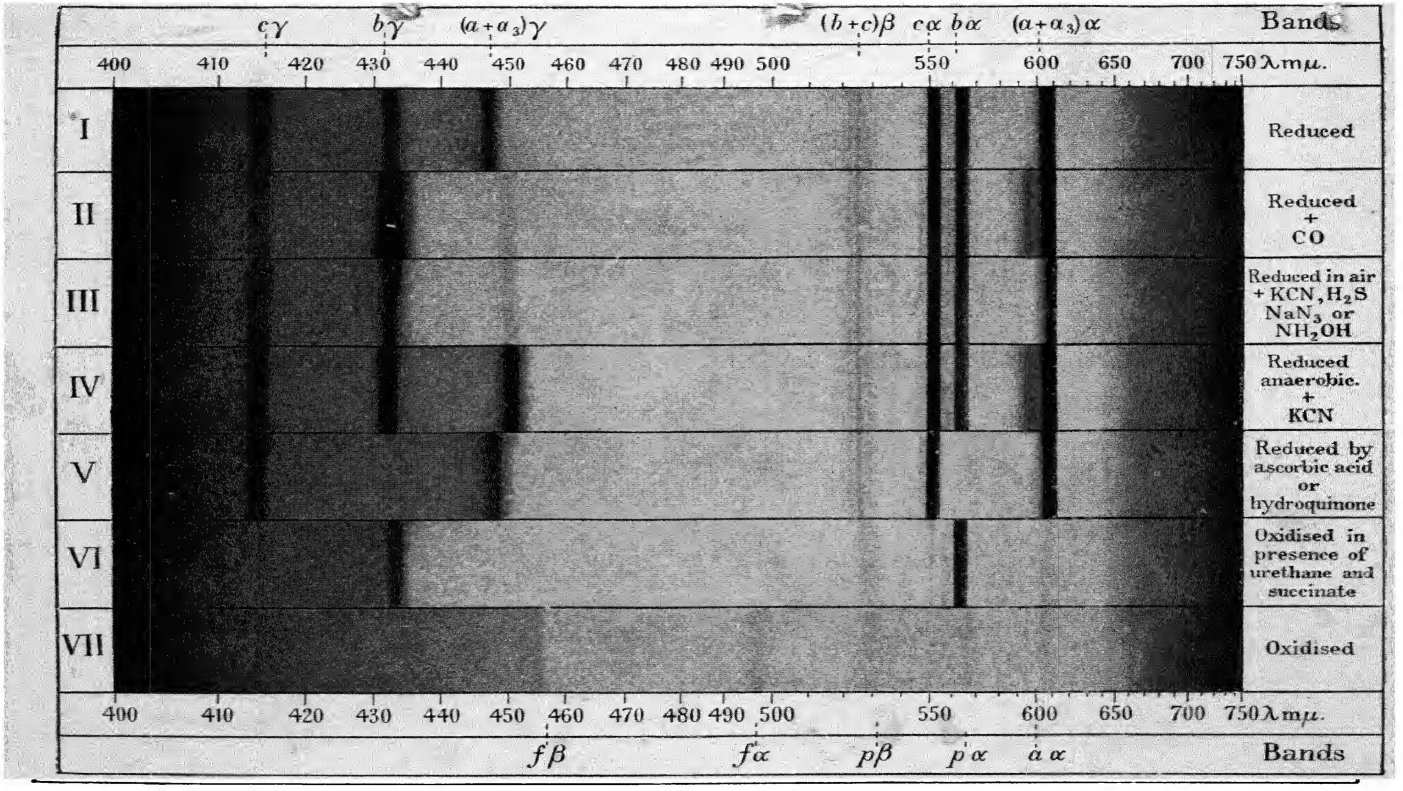


PLATE VII (see p. 202)

CYTOCHROME AND CYTOCHROME OXIDASE.

Diagrammatic representation by Keilin and Hartree. Reproduced by **permission**.

to insoluble material. The suspension shows five diffuse absorption bands in the visible:

(1) a narrow band due to a small amount of reduced cytochrome *a*,

(2) 567mμ, fused α bands of cytochrome *b* and *c* (oxidised state),

(3) 529mμ, fused β bands of the same components,

(4) 495mμ } flavoprotein bands.
(5) 455mμ }

The spectrum is not affected by cyanide nor does it change on keeping (N_2 atmosphere), so that no reducing substances are present. Three Soret or γ-bands, 448, 432, and 415mμ can easily be recorded on reduction. The presence of a succinic dehydrogenase is shown by a rapid reduction of cytochrome in the presence of sodium succinate, and on shaking with air the cytochrome soon reverts to the oxidised state. The succinic acid is

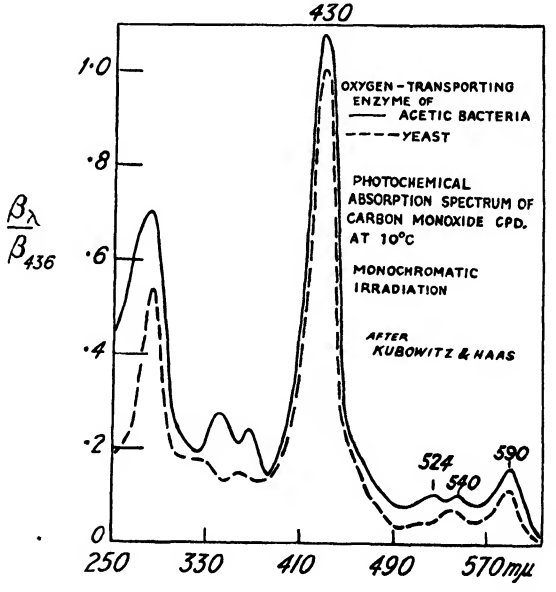


FIG. 74

oxidised to fumaric acid, which is in turn converted to malic acid through the intervention of fumarase.

The suspension now shows oxygen uptake accelerated by added cytochrome *c*. The reduced cytochrome shows as strong a band but there is an obvious deficiency of cytochrome *c*. The γ-bands are best seen by using a strong light and a filter (ammoniacal copper sulphate), and sus-

pending the preparation in 70% cane sugar solution or glycerol. These bands can also be seen in the thoracic muscles of insects.

Before proceeding further with the account of this work it is necessary to digress a little and describe the brilliant work of Warburg and his colleagues on the photochemical determination of the absorption spectrum of the respiratory enzyme.

A paper by Kubowitz and Haas (*Biochem. Z.*, 1932, **255**, 247) is important, especially from the point of view of technique concerning the photochemical method of determining the absorption spectrum of the oxygen transporting enzyme. The method involves the decomposition of the carbon-monoxide addition compound and represents a considerable advance on earlier work (*ibid.*, 1929, **29**, 64), when fifteen monochromatic radiations between 250 and 600mμ were used.

The authors used a sodium lamp, a thallium lamp (Osram) and " white " carbons impregnated with rare earths (Siemens). Most irradiations were made at 0° C. Experiments were carried out on acetic bacteria (*B. Pasteurianum*) and yeast cells (*Torula utilis*). Their respiration was reduced to *ca* 30% by carbon monoxide and restored to *ca* 60% by irradiation. From the light intensity needed for this increase, the absorption spectrum was calculated.

Acetic bacteria are more sensitive in the dark to carbon monoxide than yeast cells. Respiration of *B. Pasteurianum* falls to half when the CO/O_2 ratio is 0·5 : 1, but the respiration of *Torula utilis* falls to approximately the same extent when the CO/O_2 ratio is 10 : 1. The yeast ferment is thus the more sensitive to light.

If the enzyme activity is partially inhibited by CO, the distribution V can be written $V = \frac{n}{1-n}$ where n is the residual activity and 1-n the activity lost by CO poisoning. If on irradiation n_d (dark value) increases to n_l (light value) when the light intensity is i, the distribution changes from

$$V_d = \frac{n_d}{1-n_d} \text{ to } V_l = \frac{n_l}{1-n_l}$$

and

$$\Delta V = \frac{n_l}{1-n_l} - \frac{n_d}{1-n_d}$$

and the light sensitivity L is given by

$$L = \frac{\Delta V/i}{V_d} = \frac{\dfrac{n_l}{1-n_l} - \dfrac{n_d}{1-n_d}}{i\dfrac{n_d}{1-n_d}} \quad - - - - - - \quad (1)$$

Ratio $^{CO}/_{O_2}$ must be the same in the dark and on irradiation

but if $N_0 h\nu$ represents one molecular quantum and z_d is the decomposition constant of the dark reaction for the enzyme CO addition compound and β is the light absorption coefficient

$$L = \frac{1}{N_0 h\nu} \cdot \frac{\beta}{z_d} \quad - \quad - \quad - \quad - \quad - \quad (2)$$

and when two frequencies, ν_1 and ν_2 are used

$$\frac{\beta_1}{\beta_2} = \frac{L_1}{L_2} \cdot \frac{\lambda_2}{\lambda_1} \quad - \quad - \quad - \quad - \quad - \quad (3)$$

equations (1) and (3) are employed in determining the relative absorption spectrum. The intensity i of monochromatic radiation needed to increase the concentration of uninhibited enzyme from n_d to n_l is measured bolometrically and L is calculated for many wave-lengths. The absorption spectrum is deduced from (3) using these L values.

In equation (2) only z_d varies with temperature. If the ratio CO/O_2 and ν are kept constant

$\frac{L_{t_1}}{L_{t_2}} = \frac{(z_d)_{t_2}}{(z_d)_{t_1}}$; experimentally it is found that $L \left[\frac{\text{min sq cm.}}{\text{cal.}}\right]$ is

B. Pasteurianum -	+10° C.	3,500
Torula utilis - -	+10° C.	11,700
Torula utilis - -	+0·2°	5,200

when i is expressed in cal/cm.^{2} minute, and $\lambda = 436 m\mu$.

For optimal results V_l should be equal to $2V_d$

i.e. $$\frac{\Delta V}{V_d} = 1 \text{ and } i = \frac{1}{L}$$

A reference line must of course be chosen in order to obtain relative β values, and the Hg $436 m\mu$ blue line is convenient and $546 m\mu$ or $366 m\mu$ may be used as subsidiary standards. Finally, all the data are expressed in terms of

$$\frac{\beta_\lambda}{\beta_{\lambda_0}} = \frac{L_\lambda}{L_{\lambda_0}} \cdot \frac{\lambda_0}{\lambda} \text{ where } \lambda_0 \text{ is } 436 m\mu,$$

and the shape of the absorption curve is shown by plotting $\frac{\beta_\lambda}{\beta_{436 m\mu}}$ against λ.

Returning to the work of Keilin and Hartree on heart muscle preparation, in addition to the components *a*, *b* and *c* of cytochrome, the spectroscopic data reveal the existence of a " component a_3 " the bands of which

tend to be fused with those of *a*. The α-band is due mainly to *a* but the Soret (γ) band at 448mμ owes its origin mostly to a_3. The occurrence of a_3 may be demonstrated in bakers' yeast, in strictly aerobic bacteria and in fresh thoracic muscles of insects. It is easily destroyed by heat, solvents, acid or alkali; it is reducible, easily autoxidisable and in the reduced state forms a CO compound, with α 590mμ and γ 432mμ. The concentration of a_3 within the cell is of the same order of magnitude as that of any other cytochrome component.

Properties of cytochrome components

Component	absorption bands	mμ	
a	aα	605	insoluble, thermolabile, not autoxidisable, does not react with respiratory inhibitors
	aβ	?	
	aγ	452	(a_1, 589mμ; a_2, 630mμ in a few bacteria devoid of *a*)
a_3 (cytochrome oxidase)	$a_3\alpha$	600	thermolabile, autoxidisable, divalent;
	$a_3\beta$	?	a_3 forms compound with CO; compounds $a_3^{\cdot\cdot}$ CN and $a_3^{\cdot}$ CN known; is easily reduced
	$a_3\gamma$	448	in biological systems. Combines with H_2S, NaN_3, NH_2OH and other reversible inhibitors of oxidase activity
b	bα	564	insoluble, thermolabile, autoxidisable;
	bβ	530	cannot be isolated unmodified
	bγ	432	
c	cα	550	soluble, thermostable, can be prepared pure, is not autoxidisable at physiological
	cβ	521	*p*H; does not form CO compound or unite with KCN, H_2S, NaN_3 in oxidised state.
	cγ	415	Forms compound with NO
$a_3^{\cdot\cdot}$CO	a_3COα	590	These absorption bands agree with those
	a_3COβ	?	recorded by the Warburg school for the
	a_3COγ	432	photochemical absorption spectrum of the oxygen transporting enzyme.

It is thus abundantly clear that a haematin with a definite absorption spectrum plays an absolutely essential part in cellular respiration.

Copper-protein compounds

Traces of copper are well known to occur in various tissues and the variations in concentration which have been observed are in all probability important to pathology. The copper cannot be dialysed from a solution of plasmolysed red blood corpuscles and is therefore unlikely to be present as an inorganic compound.

Mann and Keilin (*Nature*, 1938, vol. 148) have isolated a copper protein compound from red blood corpuscles (0·34% Cu) as bluish crystals which they designate haemocuprein. The compound accounts for most of the copper present in the raw material. Haemocyanine (see below) and the polyphenol-oxidase from potatoes are also copper-protein compounds.

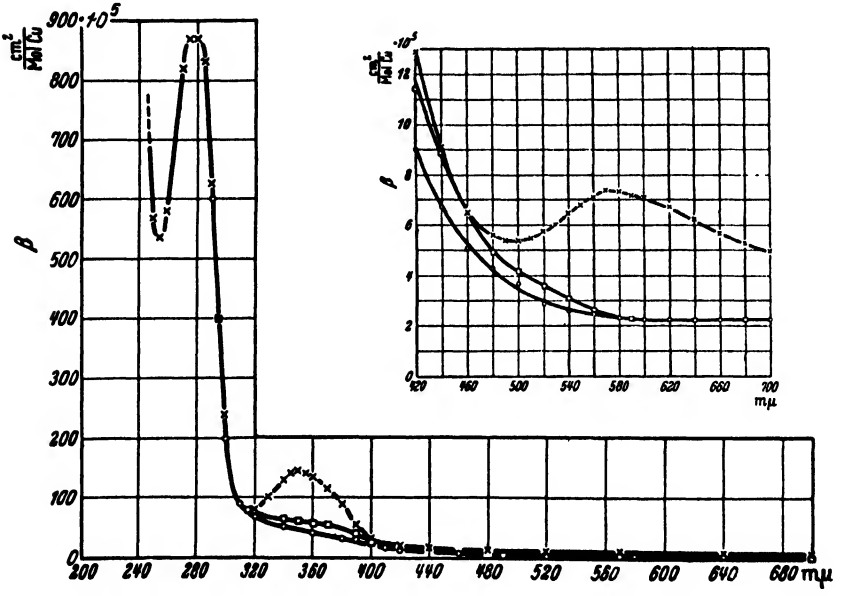


FIG. 75

Absorption spectra of octopus haemocyanine

x——x Haemocyanine solution, saturated with O_2 (oxyhaemocyanine, $\lambda_{max.}$ 350mμ.)

o—o— ditto, saturated with carbon monoxide.

□—□— ditto, saturated with argon (haemocyanine).

——— *inset* (visible region only).

x——x oxyhaemocyanine.

o—o— CO-haemocyanine.

□—□— haemocyanine saturated with argon.

After Kubowitz and Haas.

Kubowitz (*Biochem. Z.*, 1938, 299, 32) has isolated pure polyphenol-oxidase from potatoes (0·2% Cu) and haemocyanine from octopus blood. The enzyme shows a typical protein absorption band at 275mμ, but no other characteristic selective absorption. It forms a compound with carbon monoxide, which likewise shows no specific absorption bands. The same is true for haemocyanine, but this substance on oxidation shows characteristic maxima at 360mμ and 575mμ. Polyphenol-oxidase forms no stable oxygen adduct.

Kubowitz has carried out a very interesting fission and re-synthesis of the enzyme, an operation which has important implications. By treating the oxidase with HCN and subjecting the solution to dialysis, the acid and copper may be removed, leaving inactive, undenatured, copper-free protein. By restoring Cu^{++} to the protein, 56% of the activity is regained with the theoretical quality of copper and 90% with three equivalents. This experiment suggests strongly that if the " prosthetic group " of an enzyme is taken to mean a constituent which can be reversibly detached or replaced, then copper must be accepted as such, and the question is once more raised as to a satisfactory definition of "coenzyme" (see p. 186).

Haemocyanin is not amenable to test in quite the same way, but the existence of a spectroscopic " label " enables the biochemical criteria of fission to be replaced by physical criteria. Hydrocyanic acid treatment again permits the preparation of a copper-free undenatured protein fraction and the re-synthesis has been ingeniously demonstrated. The absorption bands of haemocyanine saturated with oxygen disappear when a current of nitrogen or argon is passed through the solution, but the re-formation of haemocyanine from protein and copper salt is clearly shown by the reappearance of the band at 350mμ.

It seems, however, that this cannot be the whole story. The origin of the 350 and 575mμ bands is obscure, and it seems more reasonable to assume that the real prosthetic group is complex, containing an organic residue which is less easily detached from the protein than Cu is detachable from the group itself. That much remains to be elucidated concerning the biological rôle of copper is evident from the fact that the copper plays an active part in the formation of cytochrome oxidase but nothing is known concerning the mechanism of the process.

Baumann and Stare (*Physiological Reviews*, 1939, **19**, 378) summarise the position tersely :

" Coenzymes are no longer looked upon as accelerators in any supplementary sense, but rather as essential integral members of a complicated ' bucket brigade ' transferring hydrogen or phosphate from compound to compound ; they empty their buckets and come back for more. They are ' carriers ' of both hydrogen and phosphate ".

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Small figures in brackets following a page number indicate a reference to a diagram or figure in the text.

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